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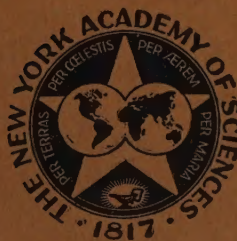
ROY WALDO MINER

VIRUSES AS CAUSATIVE AGENTS IN CANCER

BY

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ROY WALDO MINER

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Consulting Editor C. P. RHOADS

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* This series of papers is the result of a Conference on *Viruses as Causative Agents in Cancer* held by the Section of Biology of The New York Academy of Sciences, November 16 and 17, 1951.

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PREFACE

By E. D. Goldsmith

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An historical survey of the cancer problem discloses that, repeatedly, the problem of cancer has seemed on the eve of solution. Hypothesis after hypothesis has been formulated. Of these, Oberling considers but three worthy of consideration, namely: (1) irritation, (2) embryonal, and (3) the parasitic or microbic hypotheses. This monograph concerns itself with the last of these.

It was Borrel who, following his unsuccessful attempts to discover the microbic cause of cancer, first subscribed to the concept that a virus may be the causative agent in cancer. Borrel was too early; his evidence was insufficient; his equipment was inadequate. Electron microscopy, ultrafiltration, ultracentrifugation and other techniques, which we regard today as standard, were not at his disposal. In 1911, however, Dr. Peyton Rous reported, in a paper published in the *Journal of the American Medical Association*, the transmission of a sarcoma in a Plymouth Rock hen by cell-free filtrates. Much has been done in the forty years which have elapsed since Dr. Rous' discovery. Our optical, physical and chemical equipment has gone beyond our greatest expectations but much still remains to be elucidated.

The pendulum in this field, as in so many other zones of medicine, has swung often and wide. The final point of rest remains to be ascertained. We are now on solid ground in many areas, however, and it was felt that it would be profitable at this time to examine the state of our knowledge. Dr. Rhoads, by virtue of his keen, inspiring and dynamic leadership and his exhaustive grasp of the cancer problem, organized the conference which resulted in this monograph. It will do more than provide another volume to the already very extensive cancer bibliography; it will synthesize existing knowledge, catalyze present investigations, and stimulate many new researches and discoveries.

INTRODUCTION

By C. P. Rhoads

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Forty years have gone by since Rous demonstrated the transmissibility of chicken tumor #1 by cell free filtrates. Our knowledge of cell particulates and the neoplastic process has increased enormously in this period. The purpose of this monograph is to review the current status of virus work and to appraise the existing data with an eye toward future trends and eventual therapeutic or prophylactic application to cancer in man.

Darlington's remark that the term, virus, has achieved "a high professional status with doubtful credentials," should be borne in mind. In discussing the role of viruses in neoplastic growth, it is important to specify carefully the criteria employed in the use of the term. It may not be enough to invoke virus origin if a new neoplasm is caused by the injection of a cell-free extract of an old one. This does establish, of course, the importance of cellular components of less than cellular size. It does not, however, necessarily indicate that the sole cause of the neoplasm is a ubiquitous, foreign, semi-autonomous organism capable of evoking immune response. There is, in short, an important distinction to be drawn between cancer as the response to a contagious, infecting agent of conventional type, and cancer as a process of aggressive cellular growth due to the inheritance of new characteristics, consequent to the modification, by mutation, of components small and filterable, or large and non-filterable, which are responsible for old characteristics.

If the second view is adopted, and there is much in biology to suggest that it is correct, we have, in the spectrum from plastid to plasmagene to virus, an explanation of the apparently conflicting, incomprehensible data regarding neoplastic disease. No one would question the role of mutagens in carcinogenesis nor can one doubt the role of cellular fragments in the same process in many species. It is not possible, moreover, to eliminate the factors of nuclear (gene) inheritance and the endocrine system. It only becomes necessary then to postulate the mutation of cellular entities, genic or cytoplasmic as the case may be, which can confer neoplastic properties upon other cells under proper genetic and endocrine circumstances. It is clear that in some instances these entities can only reproduce and multiply within the entire cellular structure. In these cases, of course, the entire cell must be transplanted.

The cytoplasmic components of Kappa-containing *Paramecia*, CO₂-sensitive drosophila, certain yeasts, and, possibly, dendritic pigmented dermal melanophores may not be too unrelated to the mammary tumor inciter, substances described by Gross and Paschkis, or the rabbit, frog and fowl viruses involved in the neoplastic process. These matters all are of the most profound fundamental interest and importance. They pose questions of the greatest significance, however.

Let us assume the transfer of cellular components in man that are capable of effecting the neoplastic process. If control of cancer is sought, one cannot eliminate host factors, both genetic and endocrine, which play outstanding roles in susceptibility to, and continued growth of, the neoplasm. One important and encouraging development is the growing mass of evidence which suggests that these factors may be susceptible to analysis and control.

For example, we can destroy the Kappa virus of *Paramecia* selectively. We can make the *Drosophila* cell outgrow and lose its CO₂-sensitive component. We can recover in bacteriophage the marked purines of the nuclear components of the bacterial host.

Similar methods may allow us to destroy selectively cancer cells or their effective components in man and thus accomplish a cure. We may acquire the ability to analyze the endocrinologic balance of the host to determine means for cancer prevention. New information for the review of particulate materials and the cancer problem has never been acquired so rapidly nor has it been of such high quality.

THE PATHOGENESIS AND PATHOLOGY OF VIRUS INFECTIONS

By Henry Pinkerton

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The objectives of this paper are to discuss briefly the pathogenesis of viral lesions, with particular reference to neoplasia, and to describe the cellular alterations associated with the activity of a few selected viruses. Emphasis will be placed on cytological lesions rather than on inflammatory reactions, since the former are specifically related to the intracellular growth of virus particles, while the latter are secondary and non-specific in nature.

Viruses, like bacteria, protozoa and fungi, cause lesions which range from suppurative or necrotizing lesions to granulomatous or proliferative ones, depending largely on the severity of cellular injury. Neoplasia may be considered as an extreme example of the proliferative type of reaction. Other than intact tumor cells, the only transmissible agents which are known to cause neoplasia are certain filterable agents, generally accepted as viruses, which induce tumors in lower animals and plants.

Bacterial damage to tissue cells is brought about largely by toxins. Although some viruses form toxins, many viruses damage cells in a more intimate way. Nucleoprotein synthesis is diverted from its normal intracellular pathways to the formation of virus particles, and viruses compete with normal cytological components for metabolites of many types, some of which are probably intermediary in nature. Biochemical lesions of varied and subtle types are thus produced. The study of these biochemical lesions is in its infancy but the electron microscope and modern biochemical and histochemical methods promise rapid developments in this field.

The available evidence suggests that virus particles of some types do not reproduce by division, but rather by acting as models or "seed crystals" for the synthesis of new particles of an identical or similar nature. Sudden mutations occur in viruses, and there is evidence that virus particles from one species of animal, on entering cells of a different species, may change their nature somewhat because the available building blocks differ slightly from those present in the original host.

Malignant cells contain abnormal self-duplicating constituents of some type which are responsible for their continuous aggressive behavior. To deny this basic assumption is to discard what we have learned about cellular physiology. Cells do not live, reproduce, and behave in characteristic manner as individual units, but rather by virtue of genes, plasmagenes, mitochondria, enzymes, and other organoid structural components which they contain. With the exception of the evocators, or "organizers," many of which appear to be sterols, these life-giving intracellular structures are composed largely of nucleoproteins. They are all self-duplicating, somewhat virus-like, and live in complex symbiotic equilibrium with each other and with the cells in which they reside.

In a very few instances, the self-duplicating intracellular components responsible for neoplasia are filterable, transmissible, and decidedly virus-

like. In most instances, they are of completely unknown nature. When their nature is finally determined, there will undoubtedly be differences of opinion concerning their relationship to the heterogeneous group of agents which we now call viruses. When we employ the term virus, we usually imply an exogenous origin, filterability, and the ability to spread from cell to cell. There is, however, considerable doubt concerning the exogenous origin of some of the agents which we now classify as viruses (the bacteriophage, for example). Pathologists who have studied carefully the advancing edges of such tumors as rectal carcinomas find it difficult to escape the belief that the malignant change spreads from cell to cell, but there is no conclusive evidence that this is the case. If we do not restrict the use of the term virus, but apply it to any self-duplicating intracellular agent, we will conclude that "normal cells contain normal viruses," in which case, as Boycott remarked in 1933, "we had better call them something else".¹ I raise this question of nomenclature because I am sure that the participants in these discussions will not be in complete agreement regarding the exact meaning of the term virus.

The metabolic peculiarities of cells, which depend on genes, enzymes, vitamins, hormones, and other undiscovered factors, determine their susceptibility to specific viral infections. Furthermore, these same factors often determine how a particular tissue will react to a given virus. Swank and I showed, for example, that psittacosis may be converted from a latent infection, without discoverable pathological changes, to a fatal illness with tissue necrosis, simply by withholding thiamin from the diet.² Similarly, a virus which smoulders in the salivary glands of about 20 per cent of apparently normal infants may suddenly parasitize renal and hepatic epithelial cells, producing cellular alterations incompatible with the continued function of these organs.³ Duran-Reynals' demonstration, that viruses which cause typical malignant tumors under certain conditions, may, under other conditions, cause only hemorrhagic necrosis,⁴ is of particular significance. The factors responsible for such alterations in the host-parasite relationship are of basic importance and will be discussed by others.

Increasing knowledge of latent viral infection and adaptive enzyme development suggests that we must be very cautious in accepting agents such as the carcinogenic hydrocarbons as the true causes of cancers. Such agents may only set the stage (by deleting some enzyme from the cells for example) for the activation of latent virus-like agents. Cells have marvelous powers of adjusting themselves to altered conditions, and, in the process of adaptation, the malignant cell may be born. Once the malignant change has occurred, it seems to be irreversible. There is evidence, however, in the phenomenon of delayed metastasis that malignant cells may lie dormant for 20 years or more. This suggests that the malignant change has been temporarily reversed.

A virus must possess certain properties if it is to be successful as a cause of cancer: (1) It must be well adapted to life within its host cells. This means that its reproduction must be geared to that of the cells, but not necessarily as closely as that of genes, which, in normal mitosis, multiply

precisely once for each cellular division. (2) It must be of low virulence and incapable of inducing a high degree of cellular immunity in its host. This suggests that it must be rather closely related to normal cytological constituents, so that it does not behave as a "foreign" protein. (3) It must stimulate its host cells to continuous aggressive proliferation.

Many latent viral infections apparently have the first two of these characteristics, but lack the third. The Shope fibroma virus has all three for a time, but eventually loses the third. The Rous sarcoma virus satisfies all three criteria, even though it may at times go into retreat and become inaccessible.

Our inability to demonstrate the presence of virus-like agents in cancers does not exclude the possibility that such agents may be present, since the known neoplastic viruses at times become "masked" in the tumors which they cause. It is possible, moreover, that viruses may be so labile that they are destroyed in the process of filtration. Another possibility is that viruses may be so specifically adapted to their own host cells that they can develop only in them and in their descendants.

On purely theoretical grounds, then, it is possible to make out a good case for viruses or virus-like agents as causes of neoplasia, on the basis of their known characteristics. Proof of any such theory must come from experimental studies.

Until then, I see nothing unscientific in accepting tentatively the hypothesis that cancer may be caused by self-duplicating virus-like agents. Such a theory certainly leads us to perform interesting experiments, designed to alter the intracellular metabolic environment in such a way that these hypothetical agents will be unable to multiply. It appeals strongly to the imagination, and I believe the evidence supports it better than the following alternative view, expressed by Willis:⁵ "When we can say of a particular tumor (that it resulted) from the application of benzpyrene . . . we know every bit as much about its cause as we know about the cause of tuberculosis." If we knew nothing about the psittacosis virus, we might equally well maintain that thiamin deficiency was the true cause of psittacosis in pigeons. Whether or not the "virus hypothesis" eventually is determined to be correct, it seems to be a useful working hypothesis. Proponents of the virus theory have been accused of making it a religion, but I am sure that many important scientific advances have been made for the very reason that working hypotheses have been tested with religious fervor.

In considering the pathology of viral infections, it is important to realize that the initial changes are intracellular. In fact, the changes are exclusively intracellular in certain dormant infections. In necrotizing infections, inflammatory cell reaction follows 48 hours or more after the appearance of cytological changes, and its intensity depends largely on the number of cells which become necrotic. The reacting cells tend to be mononuclear, but there are striking exceptions to this, such as lymphogranuloma venereum and infectious hepatitis. Lesions are often diffuse and interstitial, because the viruses tend to grow in one type of cell, such as the ganglion cells of the brain.

The cytological changes include the formation of inclusion bodies, ballooning degeneration and cytolysis. Cells may die rapidly or may only be stimulated, in which case they show striking alterations in size, shape and staining properties. Gigantism of individual cells and multinucleated giant cells are common occurrences and it is evident that the minute particulate components of the infected cells are arranged in patterns quite different from those seen in normal cells. The ordinary light microscope shows some of these altered patterns quite clearly, and has the advantage that it allows us to observe living cells, as well as cells stained by various methods. The pictures seen will also serve as an appropriate introduction to those shown by electron microscopy.

Of particular interest is the formation of so-called inclusion bodies, some of which have been observed for more than 100 years. Since these bodies are the most obvious indication of disturbed intracellular metabolism, they are a logical starting point for the study of the complex biochemical lesions caused by viruses. It is surprising that so little attention has been paid to the study of these interesting structures by modern histochemical methods.

About 60 diseases of man are of proven or strongly suspected viral etiology; specific viral inclusions are associated with about half of these. The proportion is about the same in viral infections of lower animals.

Viral inclusions occur either in the cytoplasm or in the nucleus, a few viruses causing both cytoplasmic and nuclear inclusions in the same cell. They may be acidophilic or basophilic. In size, they range from 1 to 20 microns in greatest dimension but most often approximate the diameter of a red blood cell. They are usually spherical or ovoid, but occasionally quite irregular in shape. When situated in the cytoplasm close to the nucleus, they are often indented by the nuclear membrane or tend to curve around it. Within the nucleus, they are centrally located, and their shape frequently corresponds to that of the nucleus. They are characteristically surrounded by a clear zone or "halo". They may be single or multiple in either cytoplasm or nucleus, but are more often multiple in the cytoplasm. Evidence of associated cellular damage is present to a variable extent.

Internally, some viral inclusions are composed of discrete coccoid or bacillary bodies and others are finely granular, while those associated with the smaller viruses are, in general, homogeneous, but may contain vacuoles.

In presenting the morphology of viral inclusions, it is best to proceed from the known to the unknown. Inclusion bodies are never found in tissues infected with micro-organisms which multiply extracellularly. Certain minute cytotropic bacteria, however, as well as some of the rickettsiae, form intracellular clusters which are not essentially different from the inclusions associated with the large viruses. Intracytoplasmic colonies of *Bartonella bacilliformis*, the etiologic agent of Oroya fever, appear homogeneous when stained with hematoxylin eosin, but are seen to be composed of discreet diplobacilli when suitably stained by the Giemsa method.⁶ In the mild chronic form of Carrion's disease, or during convalescence from the acute form, this micro-organism is the cause of nodular cutaneous and subcutane-

ous lesions which microscopically appear malignant, and which continue to increase in size for several weeks, at times reaching the size of a hen's egg. Eventually, however, probably because of the acquisition of immunity, these remarkable lesions regress and completely disappear. *Rickettsia burneti*, the cause of Q-fever, forms compact intracellular clusters in the tissues of infected guinea pigs.

Spotted fever rickettsiae have the peculiar ability to grow in compact clusters within cell nuclei.⁷ This picture is diagnostic of spotted fever in the tissues of ticks, and is occasionally seen in mammalian tissues. Such intranuclear structures greatly resemble viral intranuclear inclusions, particularly when they are not stained sharply enough to bring out their internal structure. Typhus rickettsiae distend the cytoplasm of infected cells, but are never seen in clusters. These three patterns, cytoplasmic clusters, nuclear clusters, and uniform distribution, may have their counterparts in virus infected cells, appearing as cytoplasmic inclusions, nuclear inclusions, and freedom from inclusions.

Crossing the rather arbitrary line dividing the rickettsiae from the viruses, it is logical to consider next a group of viral diseases in which cytoplasmic inclusions composed of discrete micro-organisms are seen, namely, the so-called psittacosis-lymphogranuloma group. This includes trachoma and inclusion conjunctivitis, as well as psittacosis and lymphogranuloma venerum, cat-scratch fever, and a number of viral infections of lower animals not yet shown to affect man. Inclusions in this group are generally basophilic, and best seen in preparations stained by the Giemsa method, the Macchiavello method, or any other staining method suitable for showing rickettsiae. The organisms or elementary bodies forming these inclusions are embedded in a matrix which stains differentially.⁸ Whether this matrix is contributed by the organism or the host cell is not clear, but it appears most likely that it is a pool of macromolecular building blocks of endogenous origin. Homogeneous plaques, 1-10 microns in diameter, have been observed in the early stages of intracellular multiplication, and elementary bodies apparently separate from these plaques in the evolution of the mature inclusion.

Another group of cytoplasmic inclusions composed of discrete elementary bodies is that represented in man by the structures seen in smallpox, vaccinia, and molluscum contagiosum, and represented in animals by the poxes. These are basophilic at first, but later become eosinophilic, and have a matrix apparently composed of lipo-protein material, and a capsule-like membrane.⁹ When freed from their capsule and matrix, the elementary bodies of these viral infections appear to be similar to rickettsiae and bacteria in their morphology. Molluscum is a proliferative but non-invasive lesion of the cutaneous epithelium. It is not associated with inflammation. In most cases, there is eventual spontaneous regression, but lesions may persist indefinitely, and single lesions are often mistaken for epithelial tumors.

As we pass on to the homogeneous inclusions associated with the smaller viruses, we are tempted to assume that they also may be composed of very minute elementary bodies, with or without the addition of a matrix formed

from cytological constituents. It is safer, however, to reserve judgment, particularly since somewhat similar structures appear in cells after injury with heavy metals and other poisons, which will be mentioned later.

Rabies and canine distemper are associated with homogeneous cytoplasmic inclusions. Recent evidence suggests that the Negri body is homogeneous even to electron microscopy, which is surprising in view of the relatively large size of the virus.

Good examples of granular nuclear inclusions are those of herpes simplex and those associated with salivary gland virus infections. The latter are of particular interest to this discussion since the viruses of this group produce cellular and nuclear gigantism of a type somewhat resembling that seen in highly malignant tumors, although these viruses are incapable, as far as we know, of causing neoplasia. Cells altered by this virus were believed for many years to be protozoa, until Goodpasture and Talbot, in 1921,¹⁰ showed that they were derived from normal tissue cells. Huge single inclusions appear in the nuclei of infected cells, while a number of smaller inclusions are characteristically seen in the cytoplasm. The nuclei are enlarged, often to three times their normal diameter, and the cytoplasm is correspondingly enlarged. The picture is so characteristic that a definite diagnosis can be made on cytological evidence alone. These inclusions are found in human tissues as well as in the tissues of several rodents. Their study by electron microscopy and histochemical methods should be particularly fruitful. Infection with the human strain of this virus is a rather common cause of death in infants and occasionally causes fatal pneumonia in adults.

Giant cell pneumonia and measles are viral diseases in which multinucleated cells are conspicuous. In the former, both nuclear and cytoplasmic inclusions are seen.¹¹ Giant cells with inclusions are also seen in varicella infection. The distemper virus in dogs caused marked swelling and proliferation of the alveolar lining cells in the lungs with many multinucleated cells, giving a picture sometimes diagnosed as adenomatosis.

Pulmonary adenomatosis may be mentioned as an example of a neoplastic lesion in man suspected of a viral etiology. The main reason for considering a viral etiology for this lesion is its close histological resemblance to Jagzkiekte disease in sheep, which is infectious and probably caused by a virus.

Definite viral inclusions are seen in the nuclei of cells in Lucké's renal carcinoma of the frog. The Shope fibroma shows cytoplasmic inclusions, but I have only seen these in published illustrations, and I have not had an opportunity to study the remarkable cytologic changes in lymphocystis disease of fishes.

It may be mentioned that the recognition of viral inclusions requires experience, and also familiarity with various intracellular structures which may be mistaken for them. Structures most often confused with cytoplasmic viral inclusions are ingested erythrocytes and nuclear lobes of neutrophils, secretion granules, droplets of hyaline, fatty, mucoid, glycogenic or calcific nature, Russel fuchsin bodies, and even the ovoid chromosomes of certain mitotic cells and swollen Nissl bodies in ganglion cells. These

are distinguished from viral inclusions by morphology, color, location, presence of a halo, occurrence in adjacent cells and other features. Location is of particular importance. Structures which might be normal in liver cord cells, for example, would be entirely out of place in bile duct epithelium, the cytoplasm of which normally should be clear and homogeneous.

Structures most often confused with nuclear viral inclusions are fluid vacuoles, glycogen droplets, and large eosinophilic nucleoli. Although, in a few instances, there is some evidence that the nucleolus participates in the formation of viral inclusions, it is usually present and intact in inclusion-bearing nuclei.

On the whole, the evidence for the occurrence of true viral inclusions in mammalian tumors is far from convincing. Nuclear inclusions have been described in gliomas, but I have not been convinced that these structures show any marked resemblance to viral inclusions. Many of the nuclear inclusions described in human tumors are probably abnormal nucleoli. The "bird's eye" inclusions in carcinoma described years ago by Leyden are cytoplasmic. The nature of these bodies and many other structures described in malignant cells has not been established.

The nuclear inclusions seen in cells poisoned by lead¹² are definite structures, but apparently differ in certain respects from viral nuclear inclusions. They are smaller and their outlines are less sharp. It is possible, however, that they are composed of macromolecules which accumulate because some enzyme system has been poisoned.

Is the absence of viral inclusions in the vast majority of neoplasms evidence against their viral etiology? Characteristic inclusions are very common in non-neoplastic viral infections and extremely rare in neoplasms. I believe that this fact is not valid evidence against the presence of viruses in most tumors. When they contain elementary bodies, it is probable that inclusions are formed by a process not unlike that involved in the agglutination of bacteria by immune sera. Several hundred small virus particles, uniformly distributed in the cytoplasm or nucleoplasm of a cell, would escape detection by the ordinary microscope, but the same number of particles massed together might form a conspicuous inclusion. If virus multiplication is restricted in tumor cells, as we have postulated, and cellular immunity is of a low grade, we might expect viral inclusions to occur rarely. The more frequent occurrence of inclusions in certain proliferative, but not definitely neoplastic, lesions (Shope fibroma, molluscum contagiosum, warts, and animal "poxes") somewhat supports this point of view.

The only conclusions which can be drawn from this discussion are that a broad consideration of the pathogenesis of viral lesions, and a survey of the cytological changes induced in cells by viruses, suggest that there is nothing illogical in the idea that viruses or virus-like agents may be the basic causes of cancers, requiring the cooperation of nutritional, hormonal, genetic, mutational and other metabolic factors for their fruition. The dictum that "the only parasite in cancer is the cancer cell itself" needs support of more painstaking work than that done in the past.

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SIGNIFICANCE OF CELL PARTICULATES AS SEEN BY ELECTRON MICROSCOPY

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It is becoming increasingly common these days to encounter reports of electron microscope observations on tumor cells or fractions of tumor cells.¹⁻³ Almost without exception, emphasis is placed on the denser particulates that may be evident, with an implication that they are of viral character and involved in the malignancy of the cell showing them. The majority of the published micrographs show clearly that the fine structure of the cells has suffered from fixation and other procedures used to prepare them for microscopy and little, if any, attention is given to other components of the cell or to micrographs of comparable normal cells.

Special interest in these dense particulates derives, of course, indirectly from the existence of chicken tumors⁴ and the mouse mammary tumor,⁵ in which the activity of a virus-like agent can be demonstrated. This excuse, notwithstanding the present particle hunt, is reminiscent of the search for a cancer microbe in vogue some 50 years ago⁶. These particulates and inclusions of an earlier time uniformly failed to induce tumors when tested experimentally, and a similar fate may be the lot of the virus-like bodies of the present day.

Be that as it may, a steady increase in interest and a parallel improvement in the results continues in electron microscopy of tissue cells. The volume of observations by such means will certainly grow, therefore, and will make electron microscopy a standard laboratory procedure in the study of cells. With such prospects in the offing, it becomes the responsibility of those making the observations to seek, first, to interpret the electron microscope image of normal cells and to determine which submicroscopic structures are common to all cells and to what extent these vary in the normal. Studies which do not recognize these considerations seem likely to have only a very limited value.

In an attempt to live up to this ideal, we have sought in our own studies to define, first, the fundamental fine structure of tissue cell cytoplasm. We intend to review this quickly and give special attention, in keeping with the occasion and the times, to a certain species of particulate which appears as one of the more interesting submicroscopic components.

The observations have been made for the most part on various types of cells grown *in vitro*. Recent improvements in the fixation⁷ and sectioning^{8, 9} of tissues permits equivalent and even broader studies of cell structure, but thus far we have used such preparations only to confirm findings made in the cultured cells.

Procedures will be discussed very briefly. The cells are grown by standard culture techniques on surfaces previously coated with a resin (Formvar). When a satisfactory collection of thinly spread cells has grown out, they are

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freed of media by rinsing in balanced salt solution and are fixed with vapors of OsO_4 while still bathed in this solution. This is the most generally satisfactory of all fixatives. Following fixation, which may be varied from a few minutes to several hours the cells are washed in water and transferred to the standard grid for electron microscope specimens.

The low power electron microscope images of these cultured cells is not dissimilar to the light microscope image of such cells; it simply shows more structural detail. It is thus possible to distinguish, besides mitochondria and lipid granules, a complexity of dense strands in the endoplasm, a scattering of submicroscopic granules and certain small intracellular fibrils especially abundant in the cortex or ectoplasmic layer of the cytoplasm.

If these cells, or cells of a similar type, are given a longer treatment with OsO_4 , the formed bodies (the majority limited by membranes) become more sharply defined, while the diffuse and frequently fibrous components of the ground substance are removed. This extraction leaves intact what may be thought of as a membrane skeleton of the cell (FIGURE 1). The same components found after brief fixation are present in the same form and distribution. They are simply freed of their imbedding matrix and so are available for better resolution.

Cells thus prepared for study have shown consistently four components of a particulate or vesicular nature. There are usually lipid granules, marked by a great density arising from their capacity to reduce OsO_4 most actively (FIGURE 4, a). There are always mitochondria varying from spherical to filamentous forms (FIGURE 1, a). The significance of these to the cell would seem to be great since they are the carriers of the majority of enzymes involved in cell respiration¹⁰. They have been implicated in the transformation of cells to the malignant state,¹¹ but the evidence is no better than possible evidence involving other cell constituents. A third component uniformly present in these images is made up of vesicular or canalicular elements which sometimes constitute a complex reticulum (FIGURE 1, b). This material is part of the innermost cytoplasm of the cell, the endoplasm. It is referred to as the endoplasmic reticulum from its location and form. It appears to be a finely divided vacuolar system. It varies enormously in different cells in the size of its divisions and, although its function is not known, this variation reflects in part the physiological state of the cell at the time of fixation. Phase contrast microscopy provides evidence of its presence in the living cell. These three components, imbedded in a matrix of variable character, have appeared consistently in the large amount of material studied. Other inclusions, such as myofibrils, secretory granules, *etc.*, are the components by which cells are differentiated.

Less consistent than the others in appearance is a submicroscopic particulate which deserves special notice, since, more than the other structures, it seems likely to be associated with the growth and proliferation of the malignant cells. Reasons for this statement follow.

The particulate in question was first observed in significant quantities in malignant cells. We described it some three years ago¹² and have since expanded our study of its variable form and occurrence.

It may appear as a small dense granule of generally spherical form (FIGURES 2 and 3). If such granules are grouped together to represent a single species of inclusion, it must be said that the diameter variation is large, from approximately $25\text{ }\mu$ to $200\text{ }\mu$. Some of the electron density of these granules doubtless is derived from the osmium fixation but not all; since,

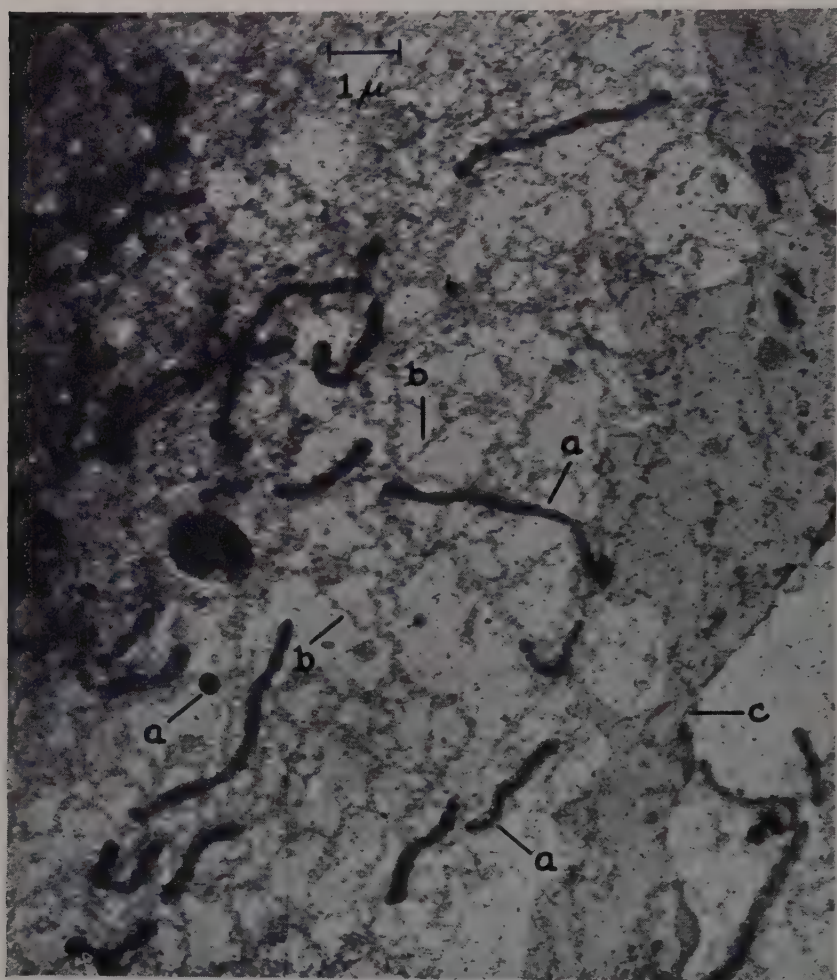


FIGURE 1. Electron micrograph of portions of rabbit endothelial cells grown in tissue culture. The overlapping margins of two cells are evident at the right. Indicated cell structures include mitochondria (a), strands of the endoplasmic reticulum (b), and the cell border (c). No lipid granules are present. All cultures in this and succeeding figures were fixed 16 hours in OsO_4 vapors and thereafter washed briefly in H_2O . Magnification: $10,200\times$.

even in tumor cells dried without other fixation, the granules are more dense than the surrounding cytoplasm. Shadowed preparations show that they retain a rotundity even in the dry state which suggests that, in the native form, they were compact bodies relatively less hydrated than the surrounding cytoplasm.

Such granules are frequently associated in pairs and show various degrees of proximity, as though in different stages of division and separation. Long tenuous strands of density and diameter similar to the granules are also often encountered (FIGURE 4). Their irregular contours and density, particulate in some regions, give the impression that they may form through the elongation and duplication of granular units which may be identical to those units which occur singly. It appears that such strands occasionally may develop to relatively tremendous lengths (FIGURE 5).

The number of granules per cell, as far as this cultured material permits observation, is not constant in any sense; only their presence in malignant

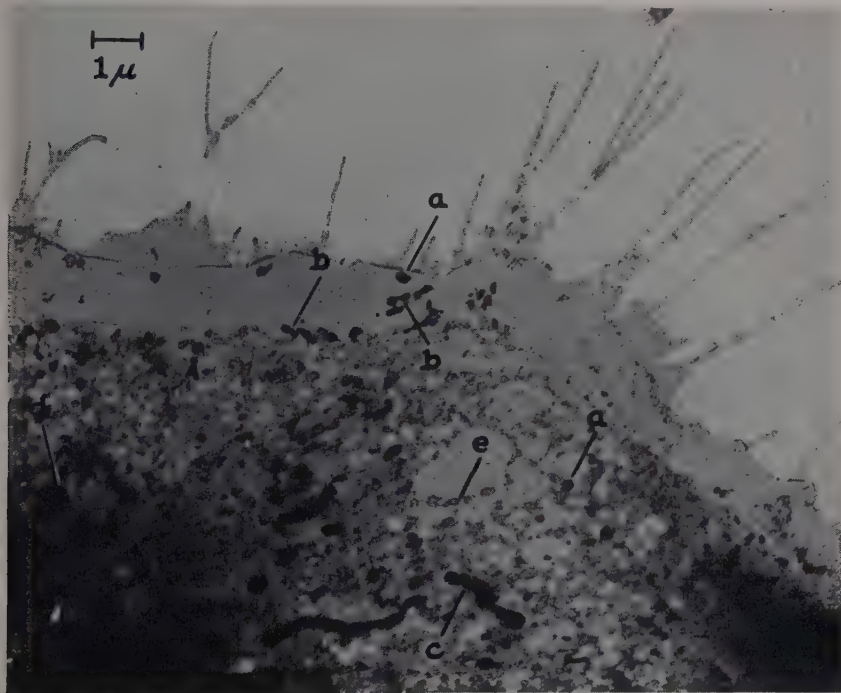


FIGURE 2. Electron micrograph of a portion of the cell shown in its entirety in FIGURE 3. The cell center is oriented toward the bottom left-hand corner. Pseudopodia extending from the cell boundary may be seen at the top. Cytoplasmic components which are evident include individual growth granules (a), paired growth granules (b), a mitochondrion (c), lipid granules (d), and a vesiculated strand of endoplasmic reticulum (e). Magnification: 8,000X.

cells is uniform. Distribution in the cell (FIGURE 3) has not followed any discernible pattern, although they are frequently found clustered at the extremities of pseudopodia. They or granules of similar dimensions, may be identified in thin sections, although the number is far less as a rule, presumably because a single section represents a much smaller part of the cell (FIGURE 6). The cells providing these observations have been derived from five rat sarcomas, both spontaneous and induced; two mouse sarcomas and one carcinoma; and a single human sarcoma.

Since our original description of these granules, two other laboratories have reported similar findings.^{13, 14} It is our feeling, however, that no one

thus far has given these cytoplasmic inclusions the exhaustive study they deserve.

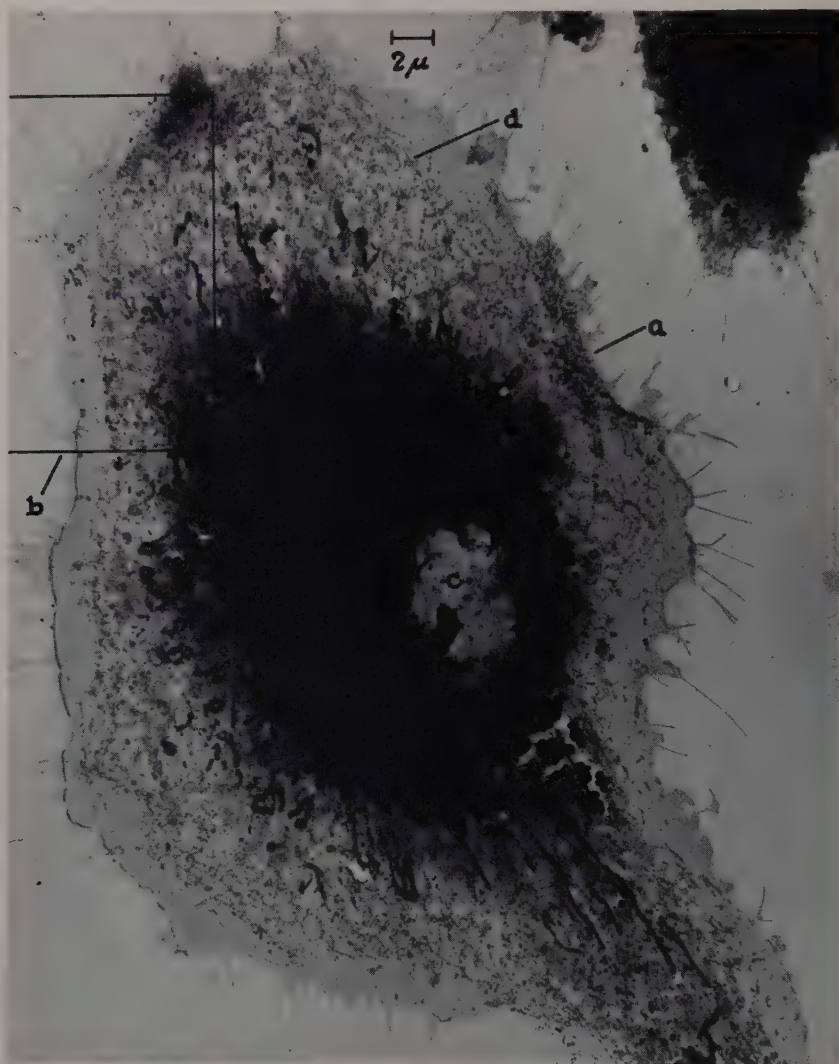


FIGURE 3. Electron micrograph of a cell cultured from a *Cysticercus* sarcoma of the rat (known as I. R. S. 4337)²¹ induced by larvae of *Taenia crassicalis*. Small growth granules are present in the cytoplasm and are particularly abundant at (a) as a cluster. Also indicated are the former location of the nucleus (c) and the material of the endoplasmic reticulum (d). The enlarged region seen in FIGURE 2 is indicated by (b). Magnification: 3,800X.

What is the nature of these bodies, and what is their role in the cell? They resemble viruses in their particulate form but this is a meagre claim to such classification. They are actually not very similar to certain specific particulates, presumably viral in nature, which were found simultaneously in cells from virus-induced chicken tumors¹⁵ and, subsequently, in the mouse mam-

mary tumor.¹⁶ These latter particulates have a more uniform morphology and size. Non-malignant cells, as originally derived from explants of adult tissue, showed few, if any, of the granules similar to those in the sarcoma cells. These normal units had been grown simultaneously in culture with the tumor cells and from tissues likely to provide cells of homologous type. Such primary cultures, however, did not grow very actively in slide preparations and the few cells obtained were mostly migratory rather than prolifer-

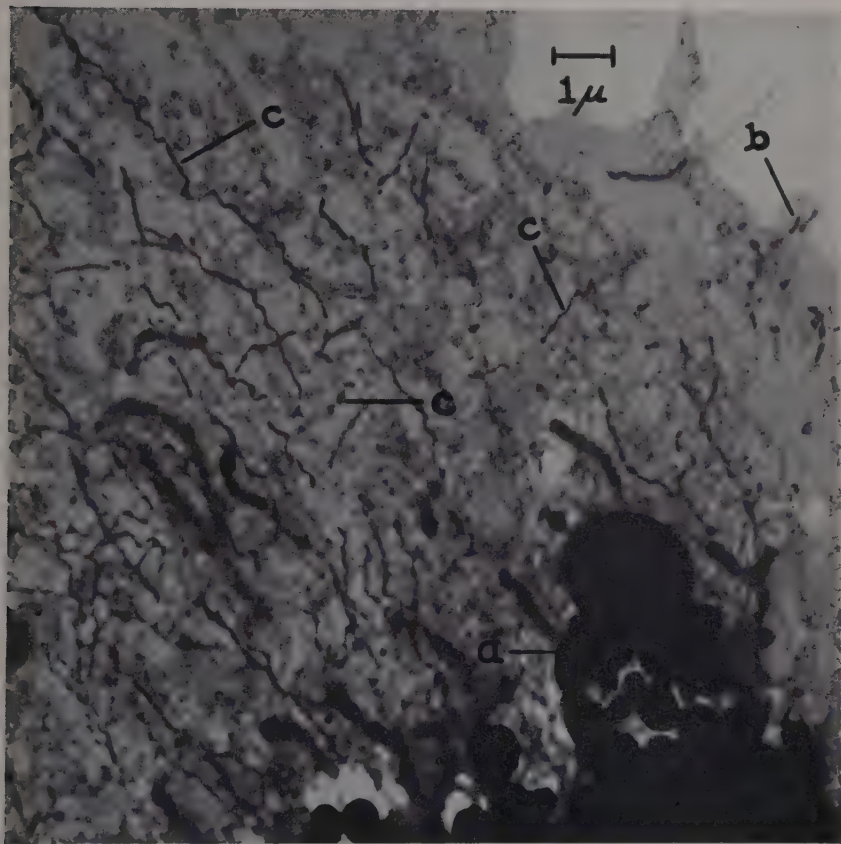


FIGURE 4. Portion of a cell from the same culture as that providing the cell shown in FIGURES 2 and 3. The paired and single growth granules at (b) are particularly interesting, as well as the long and short strands, presumably made up of growth granules, at (c). Lipid inclusions are indicated by (a). Magnification: 7,800X.

ating. We were therefore comparing rapidly proliferating tumor cells with "resting" normal cells. Similar particulates and strands were found in normal cells only when a comparison was made with actively growing cells derived from explants of very young embryo tissue (FIGURE 7). We have been led, therefore, to associate these granules with growth processes in the cell, *i.e.*, in the production of new protoplasm, and they have been tentatively referred to as growth granules.

A few correlations can be made between the occurrence of these growth

granules and light-microscope observations on neoplastic and normal cells. For example, the malignant cell has frequently been described by Ludford¹⁷ and others as more dense, granular, and refractile than its normal counterpart. It is quite certain that this is an overall effect produced in part by the concentration of these particulates. Ludford and coworkers¹⁸ have also

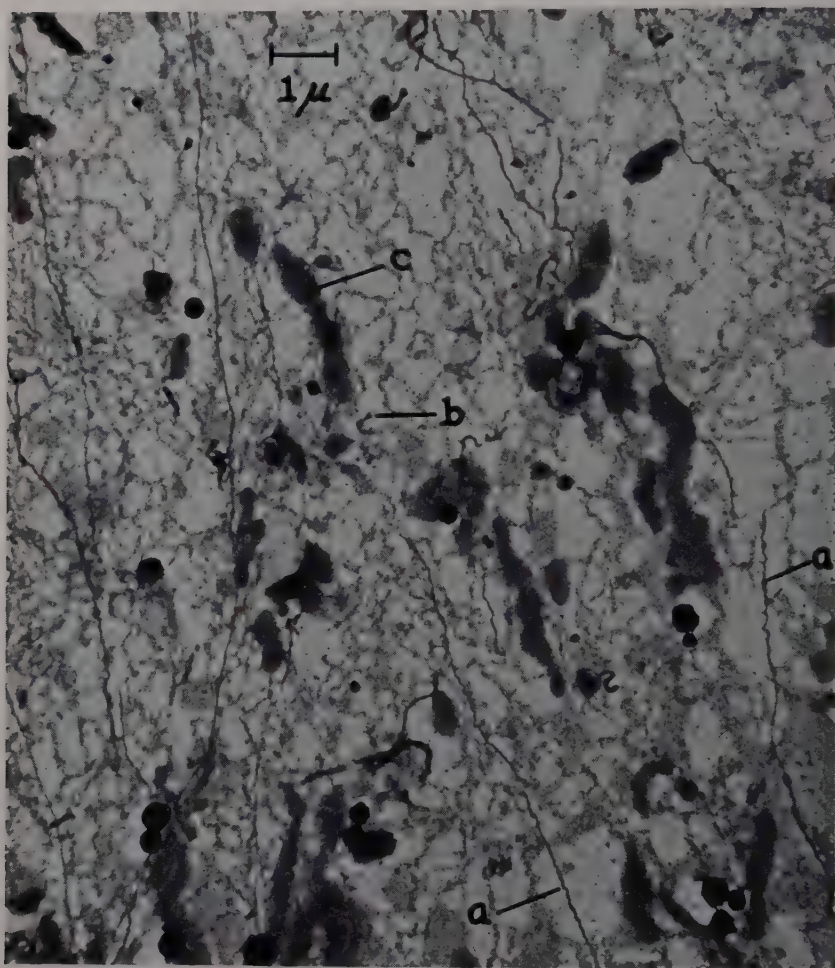


FIGURE 5. Electron micrograph of part of a Jensen rat sarcoma cell grown in tissue culture. Long filaments similar to those in FIGURE 4 are evident at (a) as well as paired granules at (b). Large mitochondria can be distinguished at (c). Magnification: 8,800 \times .

taken pictures of cultured tumor cells with ultra-violet at a wave length (around 2600 Å) assumed to be absorbed by nucleotides. The cytoplasmic densities pictured are frequently localized in what Ludford calls the cortical part of the cytoplasm where, in electron micrographs, it is common to find prominent aggregations of these particulates. This fact suggests that they may have a high content of ribo-nucleotides, which might be expected if

they are accepted as multiplying components of the cytoplasm. A suggestion of RNA composition comes also from studies of the microsomal fraction of cells in which these bodies probably belong.¹⁹ It has been reported that

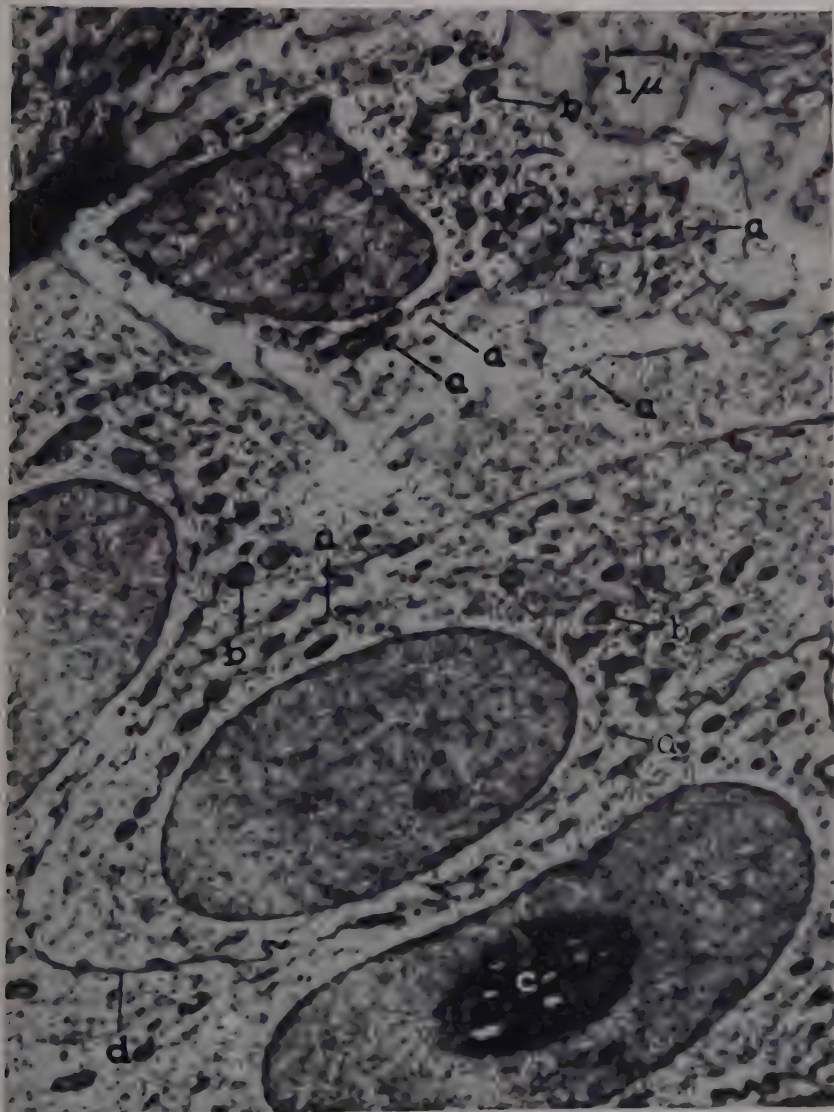


FIGURE 6. Electron micrograph of a very thin section of a Jensen rat sarcoma after fixation in buffered osmium tetroxide (Palade & Rosenberry¹⁹). The particulates of the fat that only a very small portion of each cell is shown here; the growth granules (a) are not very abundant. Other identifiable structures include mitochondria (b), the large nucleolus (c) and the cell membrane (d). Magnification: 10,200X.

RNA is concentrated in the microsomal fraction and is particularly high in the microsomal fractions of hepatomas.²⁰

It is not possible to conclude much about the role of these components

from available information. The association with growth processes seems established in part but needs further exploration. In the realm of speculation, a variety of interesting thoughts come to mind. For example, it is attractive to think of them as centers of synthesis of all cytoplasmic components. There is some preliminary evidence from the micrographs that mitochondria may begin their development in this form, but elements of the endoplasmic reticulum, the lipid granules, and inclusions, the distinctive features of differentiated cells, may be similarly derived. If such is the case,

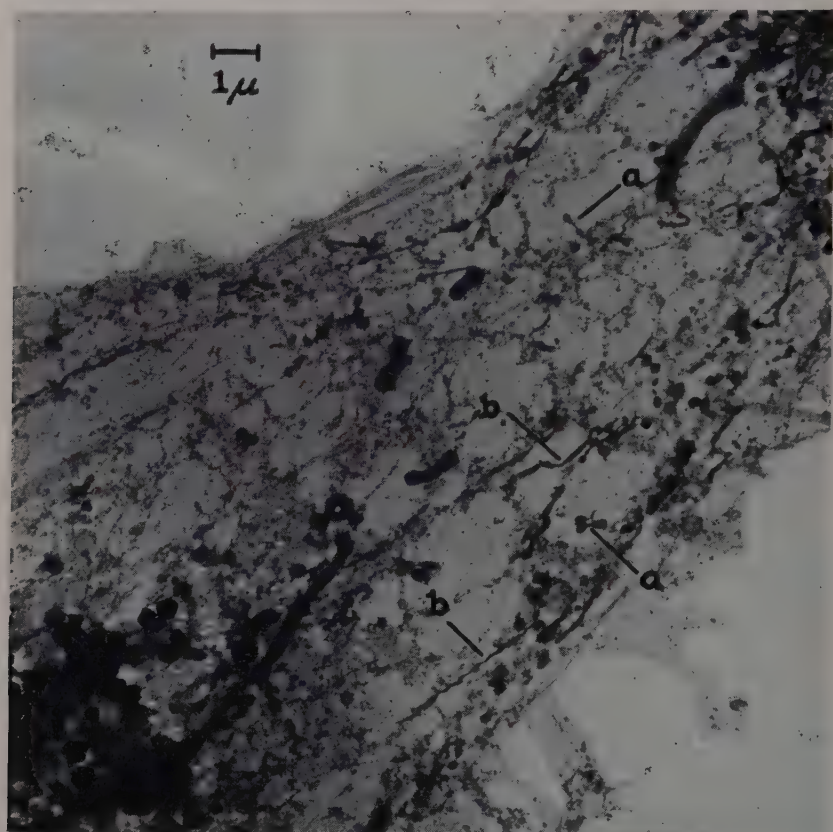


FIGURE 7. Electron micrograph of a cell, presumably myoblast grown in tissue culture from an explant of rat embryo heart. Growth granules are evident at (a) as pairs of granules and at (b) as strands. Magnification: 7,200 \times .

we are led to postulate that there are several subspecies among this class of cytoplasmic particles and that the complement of these in any cell would determine its type of differentiation to some extent. Such speculations, however, are more far-reaching than the present information justifies.

It is not possible at this time, therefore to define the significance of these granules. Their observed presence is the single feature which currently has significance, and is a prerequisite for the further study which they undoubtedly deserve. They may be of considerable importance in malignancy,

since any mutation of the normal function of centres involved in the synthesis of protoplasm, or a change in the normal complement of such centres, could express itself in the unbridled proliferation of the malignant unit.

The apparent absence of any other virus-like particle in the tumor cells studied is perhaps of as much interest as the observation of these particles. It should be noted, however, that, except for one or two cases, the tumors examined had been transplanted through many generations and could conceivably have lost any tumor inciter of viral character. Our observation may not have been sufficiently acute, however, and we have not seen what is most significant in this regard.

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CELLULAR PATHOLOGY OF VIRUS INFECTIONS AS SEEN WITH THE ELECTRON MICROSCOPE*

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There is little point in a discussion of what it is possible to see with the electron microscope in cells infected with several different viruses unless it is possible to demonstrate clearly at least two things, that we may see structures not previously apparent in the light microscope, and that these new findings bear some relationship to events in the living cell.

The first requirement is easily fulfilled by reference to the preceding paper in this monograph and by reminding you that procedures for making thin sections for electron microscopy have become almost a matter of routine during the last few years.

The second requirement, concerning "fact and artifact," is much more difficult to assess. Some confidence is placed in the procedure of short fixation of tissue culture cells with osmium by the studies of Strangeways and Canti 25 years ago,² a procedure incorporated into the technique for the preparation of cells for electron microscopy by Porter, Claude and Fulham.³

We have tested these results a little further by comparing phase and electron microscopy on the same cell and my first figures will show a comparison of a living and healthy cell before fixation with an electron microscope image after the usual brief fixation of 15 minutes in osmium vapor. This procedure has been repeated on a series of five cells.⁴

Such results have led biologically-minded electron microscopists to trust these images down to a certain size level between 500 Å and 1000 Å, although the microscope yields resolutions far below this. There are few guides beyond this point, except homogeneity, continuity of material, and reproducibility.

Peculiar inconsistencies may still occur in sectioning, such as the irregular change in the shape of the red cells when the tissue is fixed in formalin and subsequently embedded in methacrylate (FIGURE 1). The red cells appear to be normal, however, if they have been fixed with osmium before the same embedding procedure.

Three general propositions will be illustrated: (1) Certain viruses, influenza and Newcastle viruses in particular, may so alter the surface of some cells that a variety of spicules replace and/or dominate the normal microvilli. Virus may be released into the allantoic fluid through this process. (2) Certain other viruses characteristically cause a ballooning of both cytoplasm and mitochondria with subsequent degeneration of the latter. (3) Smaller viruses, such as encephalitis, may destroy cells by a piecemeal effort, with no visible general effect on the cell, and then proceed as if by replacement of the ground substance.

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The first virus carefully studied as it grew in the chick chorio-allantoic membrane was fowl pox.⁵ We used osmium fixation and embedding and sectioning by the Bureau of Standards⁶ technique in all viruses studied in this medium.

Three discoveries were made in this infection which were not either clearly seen or proven in the studies by light microscopy.⁷ First, many of the vacuoles occurring in the cytoplasm of the cells are distended mitochondria with virus nearby. The walls of the vacuoles subsequently break down and disintegrate. Secondly, virus may exist freely in the cell's cytoplasm before

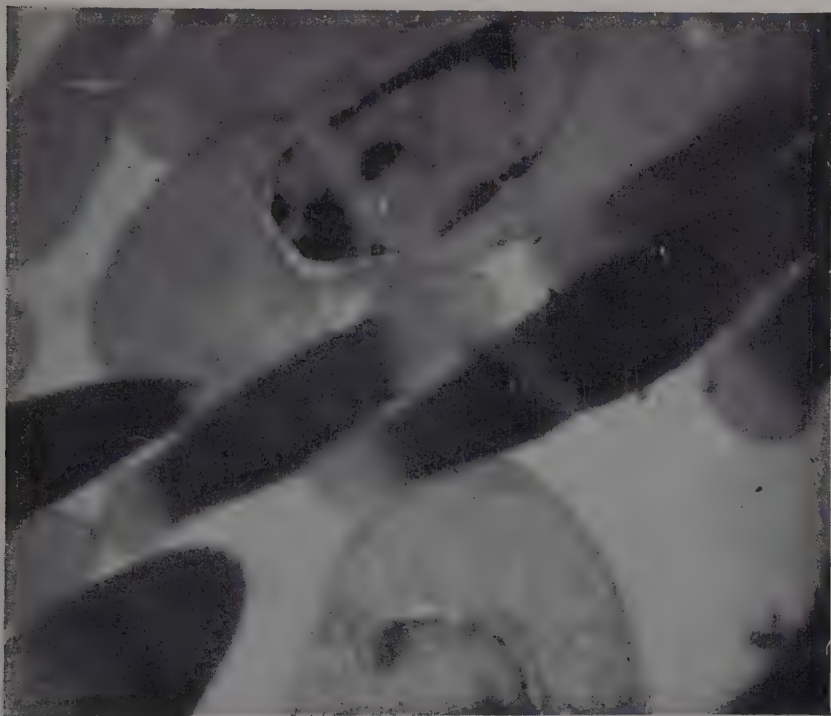


FIGURE 1. Swelling and distortion of red cells in chick chorio-allantoic membrane following formalin fixation and subsequent methacrylate embedding.

the inclusion is formed (FIGURE 2). Third, the nucleolus in infected cells may stand out as a peculiar ribbon-like or regular filamentous structure. Subsequent studies⁸ on a variety of other cells have shown that the normal chick embryo nucleolus may often show a definite structure. However, the normal chick embryo nucleolus lacks the uniform, sharply-defined, and symmetrical appearance apparent in the fowl pox infections.

Studies on vaccinia and herpes⁹ virus infections have emphasized again the effect of these viruses on the mitochondria. Vaccinia virus may be shown free in the cytoplasm, but the usual inclusion body results when great masses of virus are bound together by a matrix.^{9, 10} The presence of masses of virus

in these inclusion bodies was demonstrated some years ago by light microscopy studies of thin cells in tissue culture,¹¹ but electron microscopy makes it possible to study the thicker cells. Our studies of herpes and pseudorabies so far have revealed no early morphological changes within the nucleus, nor has the virus been recognized in the cells. Both viruses produce typical intranuclear acidophilic inclusions on the chick membrane, however.

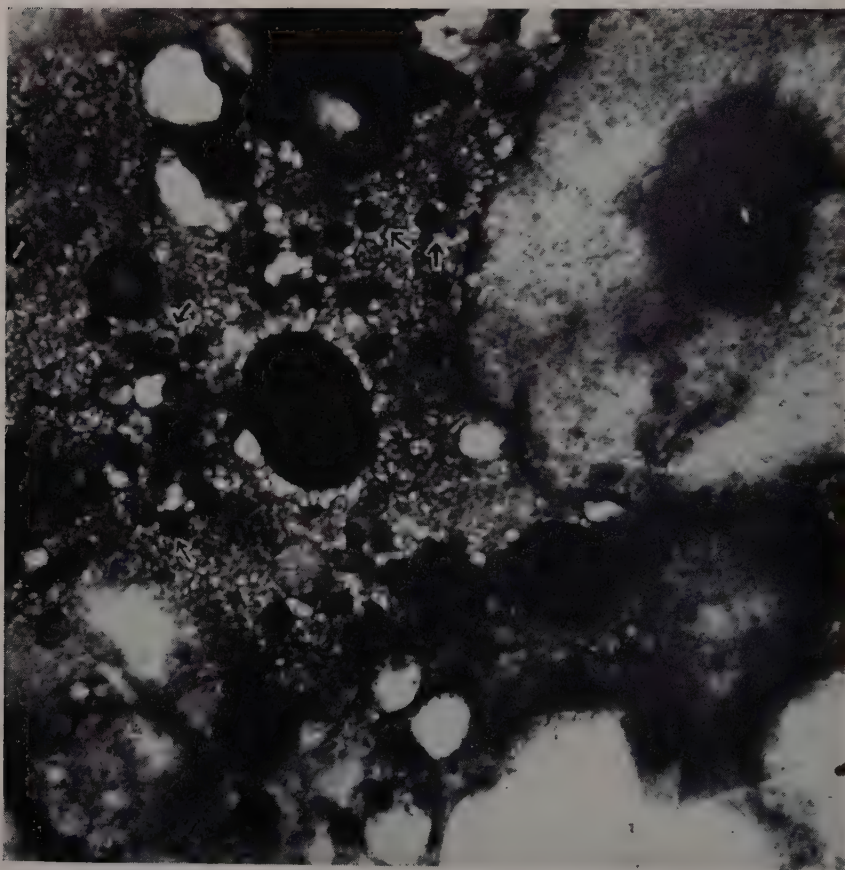


FIGURE 2. Fowl pox virus scattered through cytoplasm in epithelial cell of chick chorio-allantoic membrane. Osmic acid fixation. Section shadowed with chromium.

Interest in the growth of influenza has been great because of the demonstration of characteristic filamentous forms of apparent virus in the allantoic fluid of infected embryos.¹² Preliminary tissue culture studies¹³ showed that the filamentous and spherical forms are both apparent during the process of virus release from the cell, if not during the actual multiplication within the cell. An extension of these studies¹⁴ by two different tissue culture techniques and with additional help from thin sectioning of infected membranes has emphasized that these long thin regular filaments may be dis-

tinguished from the normal short microvilli. Filament formation with virus within the tips of the filaments takes place before breakdown of the cell, and appears with several strains. Similar observations on the projection of the filament from the cell into the allantoic fluid have been recently recorded by Wyckoff.¹⁵

The growth of Newcastle disease virus in cells has been studied in our laboratory during the last five years. A study of cells infected with the

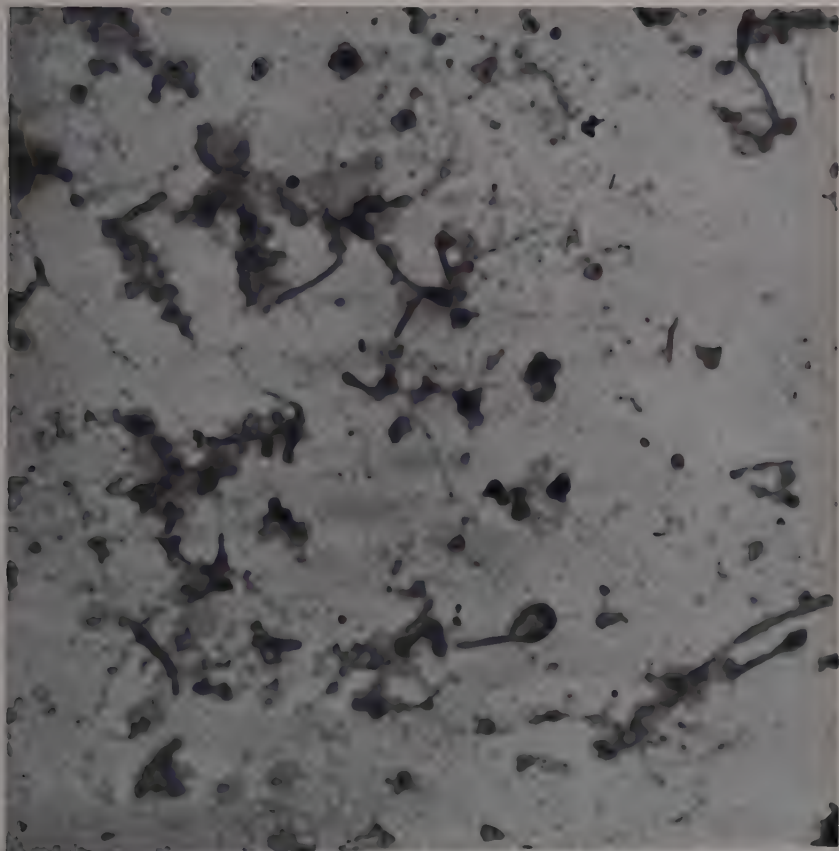


FIGURE 3. Newcastle disease virus (B strain) from destroyed chick embryo chorio-allantoic cell infected before cultivation in tissue culture. Osmic acid fixation 15 minutes. Unshadowed.

virus before tissue culture often may show stalks with ballooned vesicles at the end. There is often dense material with sharply delineated edges (FIGURE 3) within the stalk. Studies of chick epithelial cells infected in Maximow slides after the cells have grown out have shown both flattened vesicles with dense intravesicular bodies (FIGURE 4) and long thin ribbon-like filaments extending from the surface (FIGURE 5). These are apparent before cell destruction.

Roller tube cultures of epithelium show the same normal club-shaped microvilli, seen in the section of the allantoic surface of the chorio-allantoic membrane. This similarity makes the early effect of the virus on the cell difficult to study in these cells. However, the process of cell destruction by the virus can be observed easily in such cultures of fibroblasts and virus particles in these cultures may be scattered throughout the destroyed cell. Filament formation is not remarkable in these latter cells.¹⁶

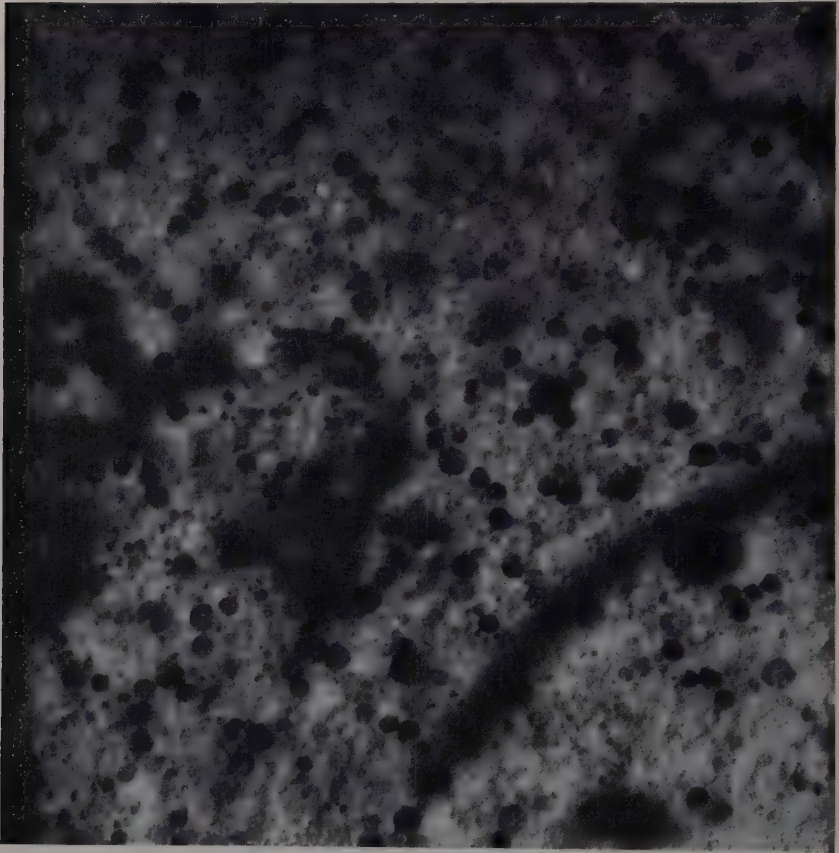


FIGURE 4. Epithelial cell from chick chorio-allantoic membrane culture. Infected in culture with Newcastle "B" 24 hours before harvest with 10^8 LD₅₀ of virus. Osmic acid 15 minutes. Shadowed with chromium.

Allantoic sac cells from embryos infected with Newcastle virus show the same formations when sectioned as those seen in the tissue cultures (FIGURE 6). Filaments projecting into the fluid are profuse (FIGURES 7 and 8) and frequently vesiculated at the end. They cover most of the cells in a 36-hour old infection. Many of these cells, however, may have been infected more recently, and inspection of cells in these small pock-like lesions, which may be seen by light microscopy, shows a continued breakdown of the cells as a

later effect of virus growth and reproduction. White cells migrating into these areas show huge ballooned mitochondria, in this case filled with granular and possibly spherical particles of the same size as the virus (FIGURE 9).

The lack of a sharply differentiated morphology for Newcastle virus prevents us from determining whether or not these particles represent virus growing within these mitochondria. It should be emphasized that epithelial



FIGURE 5. Epithelial cell from chick chorio-allantoic membrane. Infected with "Blacksburg" strain of Newcastle disease virus, 10^8 LD₅₀ while in culture. Harvested one day after infection. Osmic acid fixation 15 minutes. Chromium shadowed.

cells infected with Newcastle virus and influenza, unlike the pox viruses, do not show mitochondrial lesions.

Studies of cells infected with virus of eastern equine encephalomyelitis have been limited to tissue culture. Several years ago we showed that tissue cultures of embryos might yield such large amounts of virus that the particles could be identified readily in the electron microscope.¹⁷ More recently, such studies of a variety of cell types allowed us to describe a series of different

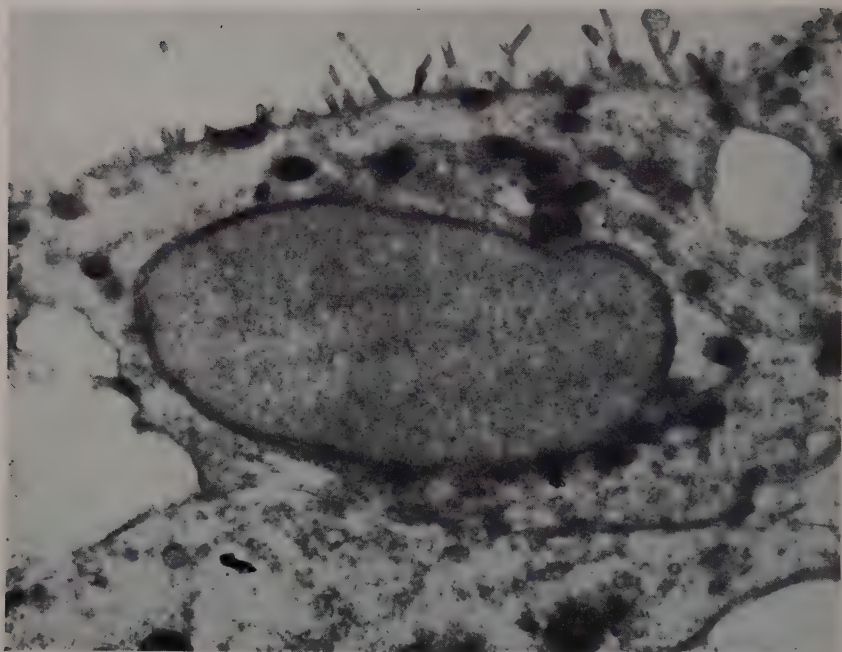


FIGURE 6. Thin section of epithelial cell infected with Newcastle disease virus ("B" strain). Most of the microvilli appear normal. A few extend into the fluid farther than usual. Osmic acid fixation. No shadowing.

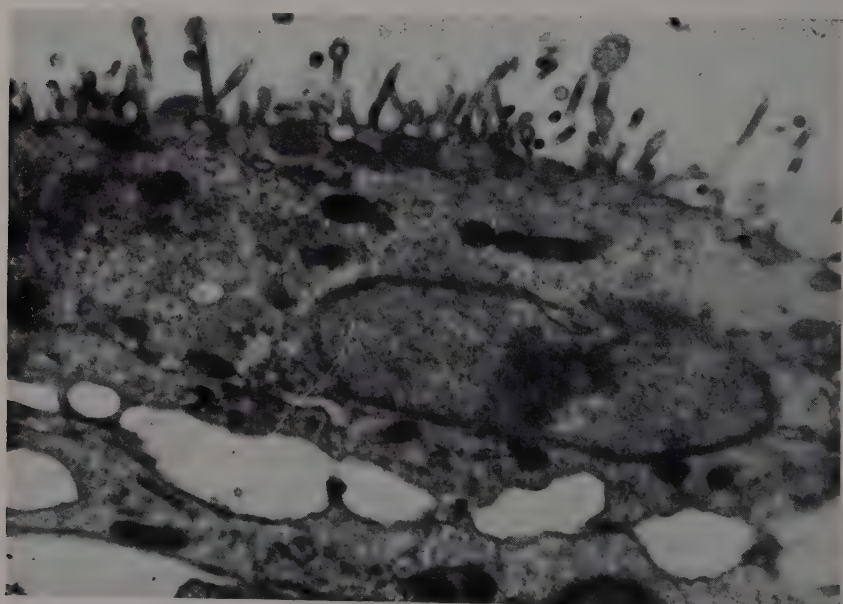


FIGURE 7. Mass of abnormal projections into allantoic fluid. Denser areas in these filaments may represent virus. Typical ballooning at ends. Mitochondria normal. Osmic acid fixation.

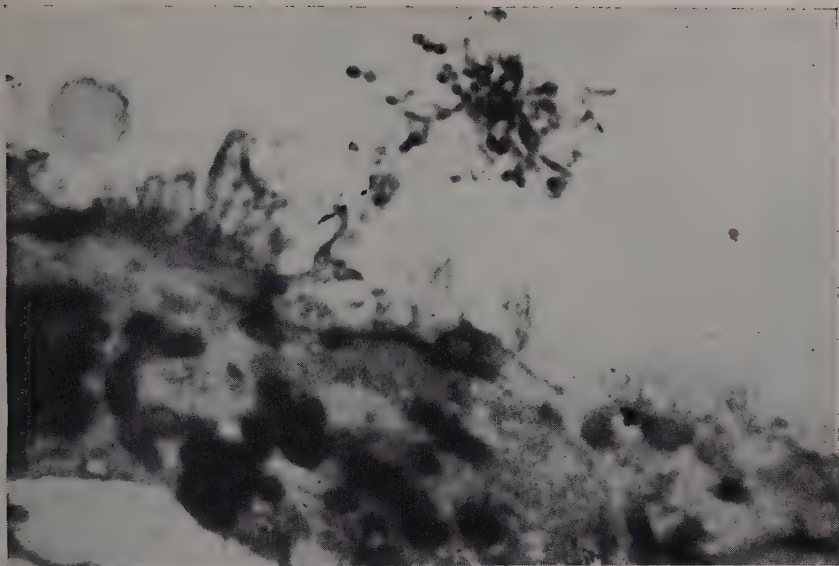


FIGURE 8. Mass of apparent virus still attached to surface of cell allantoic surface. Mixture of spherical and filamentous particles. Osmic acid fixation.

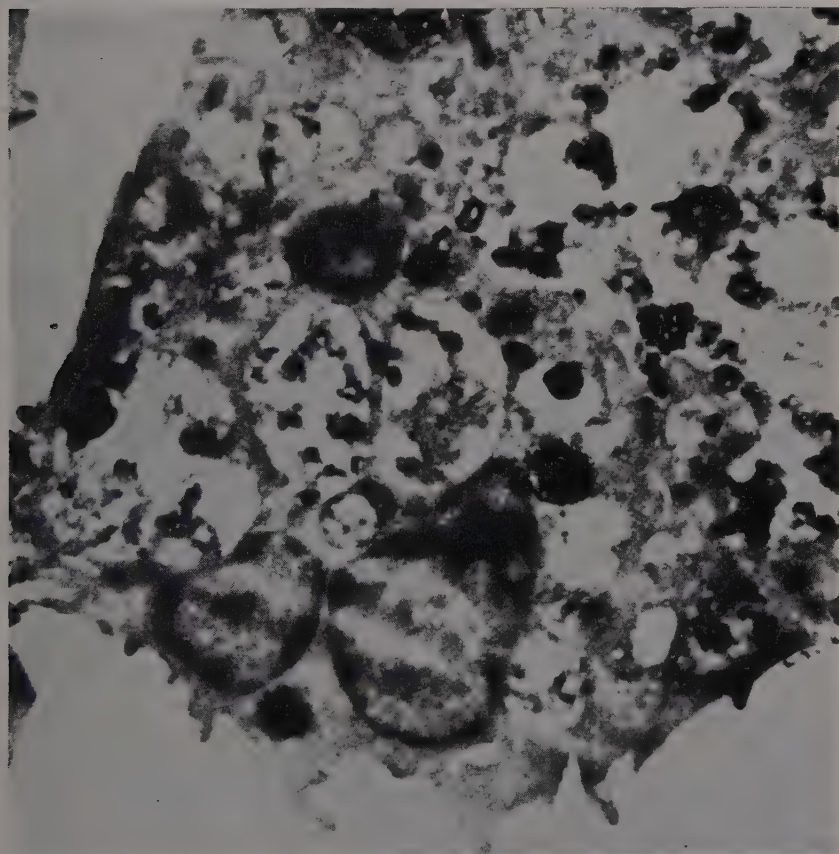


FIGURE 9. Leucocyte from "pock" on allantoic surface of embryo infected with virus of Newcastle disease. Mitochondria greatly distended and disintegrating. Granular particles within these. Osmic acid fixation.

effects of this virus on the cell's ground substance.¹⁸ A cell is seen in FIGURE 10 in a roller tube infected with virus which apparently replaced the ground substance of that cell before it fell out of the cell.

Warning is given to those who intend to interpret tissue cultures from "normal" chick embryos. These may sometimes contain particles appearing in masses,¹⁹ with sharply outlined edges and all of the characteristics of virus particles. These particles have been seen in three separate experiments in

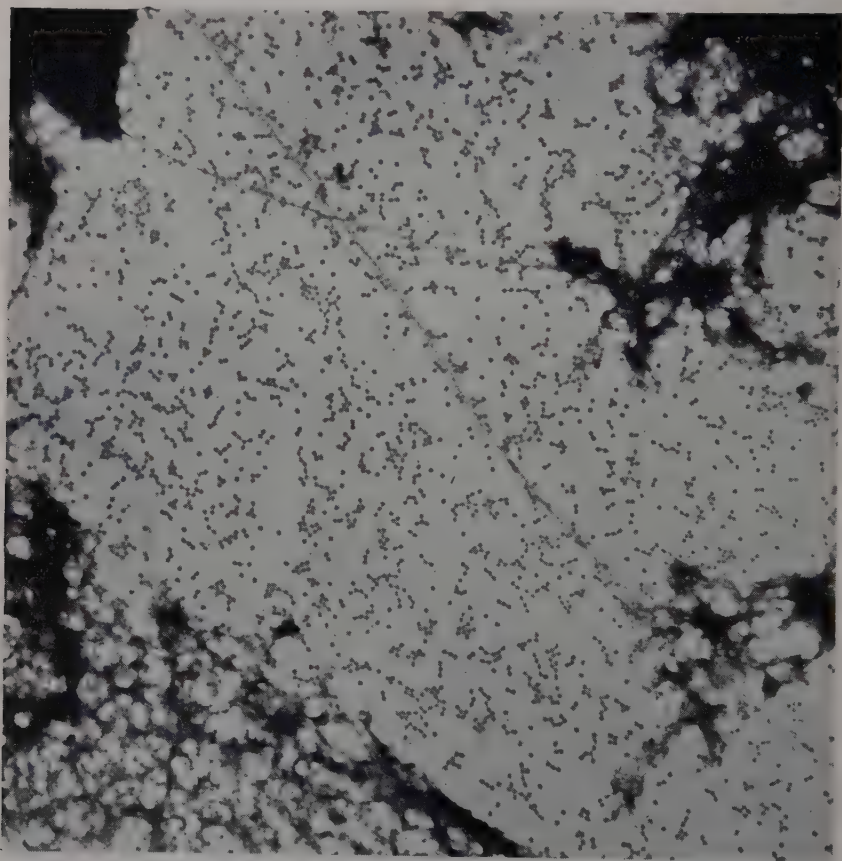


FIGURE 10. Disintegrating cell from tissue culture of chick embryo muscle infected with virus of eastern equine encephalomyelitis. Masses of virus liberated from cytoplasm. Osmic acid fixation 15 minutes.

150 different cultures. The particles are larger than encephalitis and smaller than Newcastle virus, with an average size of $70\text{ m}\mu$. They may represent a virus resident in certain embryos, such as that of fowl lymphomatosis.

Summary. Some viruses, the pox viruses in particular, are found at first dispersed through the cytoplasm. The pox viruses and herpes seem to have a marked effect on the osmotic properties of the cytoplasm, perhaps decreasing the escaping tendency of water. Ballooning and destruction of the mitochondria occur subsequently.

The influenza and Newcastle viruses stimulate the formation of surface filaments and virus presumably is liberated into the allantoic fluid as a result, before cell destruction sets in. Encephalitis destroys the cytoplasm itself, apparently replacing the ground substance at the spot. Admittedly, all three effects may vary with different cell types, as is apparent in Newcastle disease virus infections.

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THE ENZYMES OF THE HOST CELL AS A REQUIREMENT FOR VIRUS SYNTHESIS*

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In discussing the growth requirements of microorganisms, we are generally concerned with the ionic and organic composition of the growth medium, as well as factors such as the nature of the atmosphere, light, *etc.* These factors are treated in the main as elements to be acted upon; the assumption is made that the organism contains the organized catalytic equipment necessary for the transformation of the growth requirements present in the external environment.

The problem of defining growth requirements for a virus is quite dissimilar,¹ since considerable evidence exists that many viruses which have been studied in the isolated state do not have numerous enzymes generally associated with the major metabolic pathways involved in the generation of energy and the assimilation of matter.† The detailed analysis of a few cases, particularly that of the bacterium, *E. coli*, infected with the viruses T2, T4, or T6, has produced results consistent with the view that the enzymes of the host cell provide the energy derived from respiration and substances such as nucleotides and aminoacids for virus synthesis.¹ This major conception has been almost implicit in our definition of a virus for many years, a definition which tied virus multiplication to the presence of intact functioning cells.

To pose the enzymes of the host cell as a requirement for virus synthesis does not merely expand our discussion of growth requirements to include enzymes in the external environment, but affects the entire problem of the biology of viruses. For instance, we must ask how the internal structures of a large virus particle such as bacteriophage T2 can affect the host's enzymes without involving the disruption of the virus particle as a prerequisite for virus synthesis. As might have been expected, these particles are disrupted within the host cell before multiplication can begin. The unique and definitive growth requirement of viruses for the integrated enzymatic activity of the host thus establishes a qualitatively new biology for virus multiplication as contrasted to cell multiplication.

When the host cell produces characteristic virus constituents, an activity in which it was not engaged before infection, various distortions of the metabolic pattern may be sought. We may look for the synthesis of new proteins and nucleic acids which are found in the virus and for changes in the amounts of proteins and nucleic acids being synthesized, as well as for changes in the synthesis of other constituents normally found in the host cell and not in the virus. Before discussing the kinds of distortions actually observed and their

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† It must be noted that biochemists are not yet in a position to state that viruses do not contain enzymes for the specific organization of nucleic acids from nucleotides or for the specific organization of proteins from amino acids. We do not know how to test for such enzymes, or indeed if an enzymatic function is directly involved in the specific organization of these particular polymers.

possible significance, it is important to note that the phenomena to be described in this discussion are taken exclusively from bacterial virus systems. It is not that the theoretical considerations outlined above are inapplicable to tumor virus systems, but that only the bacterial virus systems present a clear picture at the present time, at least with respect to chemical aspects of these phenomena. This is due primarily to the simple methods which have been devised by the students of the bacterial viruses to insure that work is conducted with virus-infected cells. On the other hand, because it is generally agreed that this methodology is applicable to virus systems in general, it may be anticipated that its extension to tumor virus systems will clarify the biology and chemistry of these systems. For this reason, furthermore, tumor problems presumably can be clarified to the extent that they can be handled as virus problems.

The distortions of cellular metabolism are seen in their grossest aspect in the effect of virus reproduction on host division. Several types of effect have been demonstrated. In the case of *E. coli* infected by a virus of the T set, the cell stops multiplying. It is known, however, that lysogenic bacteria may multiply and grow while reproducing virus. The basic data on lysogenesis have been in the literature for many years but were neglected until recently. The re-investigation of this problem by Lwoff and his collaborators has revealed that a single cell of *B. megatherium* may divide and multiply a virus within it without self-destruction and lysis.² Only when certain stimuli, such as ultraviolet irradiation or SH compounds, are applied to the infected competent cell, competence determined by the nutritional history of the host, is a process begun in which large scale virus multiplication also begins, leading to lysis and liberation of many virus particles. The virus which is multiplied in the cell to which induction stimulus has not been applied has been detected only by the production of infectious virus as a result of these or comparable stimuli. It is considered that the virus multiplied in the pre-induction state is morphologically incomplete and is termed "prophage" by Lwoff. The multiplication of prophage in *B. megatherium*, therefore, does not appear to alter radically the growth process, processes of adaptation, or cellular division and multiplication.

It should be noted that infected *B. megatherium* may pass through two divisions after induction, before the resulting four cells break down to liberate large amounts of infectious virus. In this case, we have an intermediate type of effect reflected in the ability of virus-infected cells to divide.

Before proceeding with the analysis of these three cases, I wish to pose the following problem: Has it been shown rigorously that a cell infected with a tumor virus goes on to divide? It will perhaps be recalled that in discussing the formation of areas of proliferation on the chorio-allantoic membrane of the chick, Beveridge and Burnet³ suggest that a virus-infected cell is damaged and liberates "something" which induces proliferation in the surrounding cells. They suggest that the "something" is either virus or "more probably, growth-stimulating substances resulting from the primary damage." We do not have a clear notion of the causal relations of virus infection

and cell multiplication in the cases discussed by these writers. Until virologists are equipped with a methodology pointing to a rigorous solution of this basic problem, it will be impossible to penetrate deeper into the nature of virus-induced tumors.

The ability of a cell to grow and divide depends on the coordinated synthetic pattern developed by the cell's enzymes. In the three cases of bacterial virus systems described earlier, (1) *E. coli* strain B infected by T2, (2) infected *B. megatherium* before induction, and (3) infected *B. megatherium* after induction, the effect of infection on chemical aspects of the synthetic pattern has been studied. In all cases, no inhibition of respiration has been observed until lysis. Major disturbances, however, do appear in nucleic acid metabolism.

In growing uninfected *E. coli* and *B. megatherium*, the quantity of ribose nucleic acid (RNA) produced considerably exceeds that of desoxyribose nucleic acid (DNA), the latter being the predominant nucleic acid of the virus T2 and presumably of that liberated by *B. megatherium*. On infection of *E. coli* with T2, RNA synthesis stops completely, although DNA synthesis is markedly stimulated after a short lag. In fact, most of this DNA may be subsequently isolated in virus.⁴ In the second case, that of infected *B. megatherium* before induction, synthesis of both nucleic acids occurs as it does in the uninfected growing cell.⁵ No data are yet available comparing uninfected and infected *B. megatherium*, although the nucleic acid figures in these instances would be of great interest. On induction of infected *B. megatherium*, RNA synthesis continues, paralleling growth, but DNA synthesis stops briefly and is then resumed at a greatly stimulated rate.⁵

Briefly then, RNA synthesis continues when cell growth and multiplication continue; their inhibition goes hand in hand in these cases. DNA synthesis in these systems appears to be more directly related to the proportion of the cell's nucleic acid metabolism which has been redirected into virus synthesis. This is one part of the cell's enzymatic equipment which has been pressed into service particularly for bacterial virus synthesis. It is conceivable that the reverse of this situation exists in some plant virus systems in which the viruses contain RNA and lack DNA.

It is of interest to note that although adaptive enzyme formation is inhibited in the *E. coli*-T2 system where multiplication and RNA synthesis are prevented, enzymatic adaptation can occur⁵ in other induced lysogenic systems where multiplication and RNA synthesis continue. This need not mean that adaptation and protein synthesis depends on RNA synthesis but, conversely, that infection may determine primarily whether host protein may continue to be synthesized.

If we were to consider the gross aspects of phosphorus and nitrogen metabolism or glucose utilization in the *E. coli*-T2 system, it might appear that virus multiplication merely involves the normal qualitative and quantitative pattern of synthesis of cell constituents which are funneled off to form virus instead of host. Although this seems to be roughly true for the amounts of N and P taken in and the amount of substance processed, the division of labor of the enzymes within the cell has become quite different. For example,

the cell's enzymes are now catalyzing the production of far more desoxyribose and thymine than was previously needed, and other enzymes are not being called on at all.

The key biochemical problem of viral parasitism may then be posed. How does virus infection produce this distorted enzymatic activity, apparently stimulating the activities of some enzymes and inhibiting the activities of others? Several possible answers come to mind. We can imagine that in an integrated system of enzymatic equilibria which obey the law of mass action, the precipitation or other form of binding of products in virus constituents would merely shift the equilibria in the direction of the synthesis of virus constituents, removing compounds from other reactions not leading to virus; or, secondly, we may say that reactions other than those essential for virus synthesis are actively inhibited by portions of the virus. A more vague variant of this hypothesis suggests that the infection process so disrupts the integrated character of synthesis that unnecessary enzymatic reactions are separated from the main body of still-integrated reactions.

In the field of amino acid synthesis and utilization, I consider the first hypothesis to be most likely. We are concerned here with about 20 amino acids and hundreds of interdependent synthetic reactions. In the utilization of tryptophan for T2, T4, or T6 synthesis by *E. coli*, we have shown that we are concerned with quantitative differences among the viruses and not with all-or-none requirements.⁶ The possibilities for the applicability of the second set of hypotheses appear exceedingly remote, therefore. The observed quantitative differences are probably associated with the different amounts of tryptophan in these viruses.

The rate of withdrawal of tryptophan to virus protein from the generated metabolic pool within the cell may determine the rate at which tryptophan is synthesized. It is conceivable that no effect would be produced on the tryptophan-synthesizing systems, in which case the rate of synthesis of tryptophan should be independent of infection or the type of virus being synthesized. In the case of thymine synthesis and utilization, however, it would appear that the productivity of an enzyme system can be considerably changed.⁷

The hypothesis of the mere displacement of enzymatic reactions in the direction of virus synthesis does account at the present time for many observed phenomena. Several observations in the bacterial virus systems, however, compel us to invoke the inhibition hypothesis as well. The pathways of metabolism leading to ribose phosphate and desoxyribose phosphate formation will serve as models for our present thinking on this question.

In order to understand the nature and cause of the shunt in P utilization and nucleic acid synthesis in infected *E. coli*, we undertook to unravel the origin of the nucleic acid pentoses several years ago. Our results have demonstrated the existence of alternate pathways of glucose-6-phosphate metabolism, as presented in FIGURE 1, in *E. coli* and many other cells. One of these pathways, which we call the oxidative pathway, leads most directly to the formation of ribose phosphate.⁸ By taking advantage of the irreversibility of the step from glucose-6-phosphate to 6-phosphogluconate, and

the adaptive transphosphorylation of the sugars of this pathway (other than glucose, *e.g.*, gluconate) by *E. coli*, we can study the ability of this pathway to support growth or virus synthesis, meanwhile bypassing the anaerobic portion of the Meyerhof pathway. It has been found that this enzymatic pathway is capable of supporting growth and ribose nucleic acid synthesis readily, but is unable to maintain a stimulated rate of DNA synthesis during virus synthesis. Virus synthesis is proportionately less.

Both pathways are operating during growth on glucose, about $\frac{1}{3}$ of the glucose passing *via* the oxidative pathway, the remainder presumably *via* the anaerobic scheme. This estimate was obtained from studies with C_1 -labeled glucose.⁹ When cells are synthesizing virus, there is no gross change in the quantity of glucose assimilated, nor of O_2 consumed and CO_2 produced from this glucose. Nevertheless, by following the fate of the isotope from

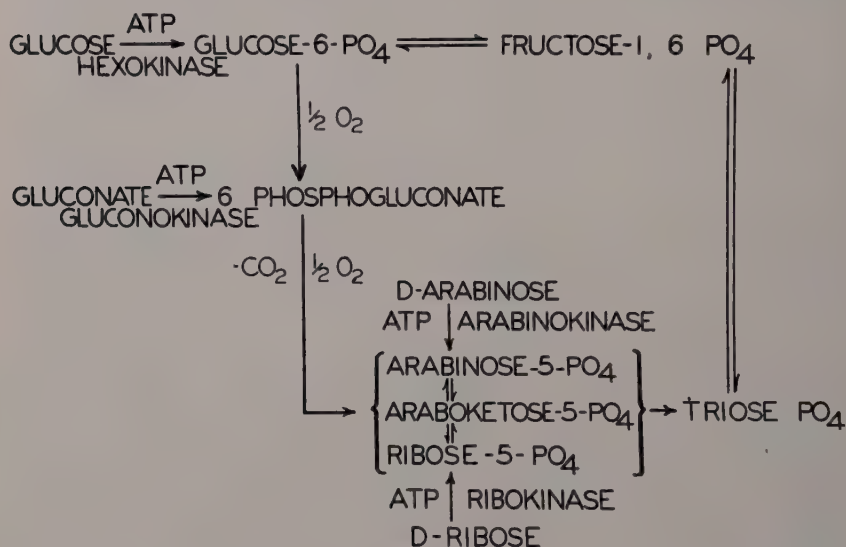


FIGURE 1.

C_1 -labeled glucose, we have shown that much less glucose passes down the oxidative pathway. Thus, the utilization of the anaerobic pathway is correlated with the synthesis of virus desoxyribose nucleic acid. It has been shown recently that desoxyribose phosphate is derived from triose phosphate and acetaldehyde.¹⁰ The anaerobic pathway gives rise to 2 mols of triose phosphate per mol of glucose, in contrast to the oxidative pathway, which gives rise to only one mol of triose phosphate.

These results indicate that the transformation in nucleic acid synthesis and P utilization in virus synthesis, as compared with growth, is functionally related to the mode of utilization of the primary phosphorylation product of glucose, glucose-6-phosphate. How is this utilization determined? At first sight, we could well imagine that in these reactions of carbohydrate metabolism, whose reversibility and dependence on the law of mass action are

well known, the diversion of glucose-6-phosphate from the oxidative or ribose phosphate pathway could readily be accomplished by the simple removal of triose phosphate to form dextroxyribose phosphate in virus DNA. The following phenomena suggest that this answer is incomplete: (1) in *E. coli* infected with ultraviolet irradiated virus T2, all nucleic acid synthesis is inhibited, although protein synthesis, P assimilation, and nucleic acid base formation continue;⁸ (2) Herriott has reported that in *E. coli* infected with ghosts of T2, devoid of the nucleic acid constituents within the head of the virus, nucleic acid synthesis is inhibited;¹¹ and (3) in a strain W of *E. coli* we have studied recently,¹² infection with T2 stops growth and all nucleic acid synthesis without virus synthesis in any medium tested.

Thus, although DNA synthesis does not occur in these three cases for what appear to be three different reasons, the equilibria are not shifted to increased RNA synthesis. RNA synthesis is totally inhibited in every *E. coli*-T2 case, regardless of the extent of DNA synthesis. We consider it likely that, in this system, we are concerned with an active inhibition of one or more steps essential to RNA synthesis. By this mechanism, not only could we account for the basic parasitic phenomena observed, *i.e.*, the inhibition of all substances which may be structurally associated with RNA, but, in addition, more of the carbon and phosphorus of glucose-6-phosphate is made available for virus synthesis. Although there is no reason to invoke a comparable inhibition in normal lysogenicity before induction, it may be suggested that the induction stimuli are inhibitors of critical reactions which throw the cell's metabolism into the synthesis of constituents primarily useful in virus synthesis.*

These questions have been raised in such detail, not only because these matters are in our experimental focus at the present time, but also because they may be related to the problems of tumor formation. In the formation of some tumors, an adult cell which would normally have ceased the production of DNA is induced to divide and produce DNA. This phenomenon goes hand in hand with the well-known distinctive metabolic pattern of tumor cells, a high rate of aerobic glycolysis. This phenomenon is conceivably accounted for by an inadequacy in the function of the oxidative pathway which then would result in the anaerobic doubling of triose phosphate formation. Is it not possible that the induction process, whether by tumor virus, butter yellow, or other carcinogen, produces inhibitory effects analogous to those described above? Although this hypothesis may be incorrect when applied to tumors, the work with the bacterial viruses has supplied some of the biological and chemical tools, so that it may be tested readily.

In turn, now that we have pin-pointed carefully the site of the distorted metabolism in a bacterial virus system, we must undertake to pin-point the site of the inhibition and its mechanism. I have mentioned this to assure you that there is no dearth of critical problems to be solved in bacterial virus

* It is being felt increasingly that lysogenicity is a more common phenomenon in nature than the extreme type of viral parasitism exemplified by the *E. coli*-T2 systems. The development of an inhibitory mechanism in these virus systems leads to total destruction of the host and, therefore, the eventual destruction of the parasite, if other compensating devices are not developed. It is of interest that, in the *E. coli*-T2 systems, an r^+ virus, which has still another inhibitory property of slowing down lysis of an already infected host, outgrows an r virus, which lacks this particular inhibitory property, when both are grown in mixed culture. The r^+ character would appear to be such a compensatory device of ecological significance.

systems and that our efforts in this direction seem to be only another aspect of the very difficult tumor problem.

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BIOCHEMICAL RELATIONSHIP OF VIRUSES WITH CELLS*

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The biochemical relationship between host cell and virus embraces a wide range of phenomena. Since some of these will be, or have already been, discussed by other authors in this monograph, I shall confine myself to certain portions of the field. More particularly, I should like to examine our information in regard to the following three questions:

(1) Does a comparison of the chemical and physical properties of the virus and host cell permit any deductions as to the origin, biological nature, and mode of replication of the virus?

(2) Is information as to the origin of the various chemical constituents of the virus particle valuable in this respect?

(3) Does knowledge of the fate of the infecting particle throw any light on our understanding of the problems of virus infection and replication?

First, however, I should say that my own experience with the virus-host cell relation has been confined to participation in a study of the biochemical properties of the coliphages and, although I shall endeavor to include some information concerning plant and animal viruses, I must disqualify myself as an expert in these two latter fields.

It is a matter of some interest that in spite of the number and variety of virus diseases, their plant and animal hosts are restricted in distribution. Only the Angiosperms of the Spermatophyta are known to be parasitized,¹ and parasitism of a second plant phylum by viruses, the bacteriophages, is limited to two orders: Eubacteriales and Actinomycetales of the Thallophyta. Among the animals, a limited number of the insects are affected, and in the vertebrates, the principal hosts are mammals and birds.

In the case of the morphology of the viruses, we again observe a limited range comprised, according to Beard,² of (a) the coccoid types (spheres, spheroids, biscuit forms, or very short rods); (b) bacilliform types (rods of ordinary proportions, filaments, or long thin rods); and (c) the sperm or tadpole types (modified spheroids with tails or modified bacilliform bodies with head structures). The plant viruses, with one exception, are spheres or long rods. The bacterial viruses are spheres or tadpole-shaped entities, while the animal viruses vary from spheres to sperm shapes. The insect viruses, with a possible exception, are bacilliform. No tadpole or sperm-shaped form affects either the insects or the plants, and no bacilliform virus parasitizes either the mammals or the birds.

The chemical composition of viruses, however, shows no inclination to stay within limited bounds. All viruses appear to contain protein and nucleic acid and, in the plant and insect viruses, these apparently constitute the whole of their chemical composition. The amount of the nucleic acid (of the RNA type) in plant viruses is usually not large, although the virus of to-

* Aided in part by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago and the National Foundation for Infantile Paralysis, Inc.

bacco ring spot contains 40 per cent and the virus of turnip yellow mosaic contains 28 per cent RNA. The viruses of the silk worm and gypsy moth caterpillar contain only DNA; neither plant nor insect virus containing fat, or carbohydrate in excess of the amount present in nucleic acid. The various bacterial viruses are remarkably similar in constitution and all of them seem to contain relatively high amounts of nucleic acid which in turn appears to be principally, if not completely, of the DNA type. There are, however, small amounts of lipid material present in the bacteriophage particle.

Whereas the plant viruses appear to be simple RNA proteins capable of isolation as pure chemical substances in so far as criteria of purity can be applied to such complex material, the animal viruses are undoubtedly more complex. They may contain either RNA or DNA, or both together, with lipid material which may range from 1.5 per cent with the Shope papilloma to 54 per cent in equine encephalomyelitis virus. RNA has been reported in the viruses of equine encephalomyelitis, Rous sarcoma, poliomyelitis, louping ill, and in the milk factor responsible for mammary cancer in susceptible strains of mice. DNA appears to be present in the virus of vaccinia, rabbit papilloma, and psittacosis. In the case of vaccinia virus, digestion with pepsin removes three-fourths of the virus substance, leaving a central body consisting of DNA. This can be removed with desoxynuclease and appears to have the attributes of a nucleus, although it is of a much smaller size than the cell nuclei.

Using the ultraviolet adsorption technique, Hayden³ has studied the point of attack on the cell of a number of viruses of the neurotropic variety. The viruses of poliomyelitis and louping ill, both ribose nucleoproteins, attack the heterochromatin parts of the cell and cause a disturbance in the very early stages of protein production. In rabies, neurovaccinia, and verruca vulgaris, the point of attack appears to be in the nucleolar stage of normal protein production.

Taken by themselves, these data do not lead very far in the direction of a decision regarding the nature of the virus particle and the process of virus replication. The chemical complexity of the animal viruses is so great that one can infer a degree of biological organization which would reduce the role of the host cell to that of supplying nutrient material, much as a mammalian tissue would nourish an invading microorganism. Indeed, with the large viruses of the psittacosis group, changes in size, staining properties, and number of intracellular viral particles have suggested to some the existence of a life cycle, although many facts related to other animal viruses do not support such an idea. With the bacterial viruses, the analogy between the high DNA content of the phage and the DNA-containing chromatinic bodies of the bacterial cell suggests a relationship which is further emphasized by the observation that drastic alterations in the chromatinic bodies are the outstanding morphological changes during virus infection. The compositions of DNA from host and bacteriophage, however, are strikingly different, and it is apparent that a bacterial nucleic acid could not be transferred directly into viral particles; although it seems probable, at least

with the coliphages, that virus synthesis is associated with the normal nucleic acid metabolism of the host cell.

Let us consider our second question (see page 909), and examine the data relating to the origin of the various chemical components of the virus particle. Our most exact information in this respect stems from experiments in which stable or radioactive isotopes are used to label the materials being transferred. In the case of the animal viruses, the technical difficulties involved in isolating the virus-host cell complex from the environment, and the difficulty of eliminating secondary or unrelated reactions in the host, have so far prevented experiments of this type, although Graham and his co-workers have succeeded in introducing radioactive phosphorus into the influenza virus.⁴ In the field of plant viruses, Meneghini and Delwiche⁵ have studied the uptake of N^{15} by tobacco plants inoculated with tobacco mosaic virus, the N^{15} being present as ammonium chloride. Under these circumstances, they have concluded that in the host tobacco plant the tobacco mosaic virus is formed from "some nitrogenous compound or compounds" such as amino acids, which undergo a more rapid exchange of N with the ammonium ion than does the extractable protein of the plant cell. Further, after infection has run its course in the plant, and large quantities of the labeled virus are present, it is found that the virus behaves as a foreign protein and is not in equilibrium with the other cell constituents. It would appear, then, that there is no evidence here of a preformed normal cytoplasmic compound of the plant being converted into the virus, but rather that a considerable portion of the virus is synthesized from the small molecular weight compounds which are available to the plant for the normal process of cellular growth.

This applies also to the coliphages of the T_1 to T_7 series, which have been most thoroughly studied in this respect; a group of viruses which has been thoroughly characterized, both biologically and physically. Information regarding the group is summarized in TABLE 1.⁶ The even-numbered phages, T_2 , T_4 and T_6 , are serologically related and morphologically similar, being tadpole-shaped particles with a head of about $65 \times 80 \mu$ and with a tail of about $100 \times 20 \mu$. This morphological similarity apparently also extends to the origin of the chemical components of the viral particle, since it has been shown by Cohen⁷, and by Kozloff and Putnam⁸, that 70 to 80 per cent of the P of the T_{2r+} , T_{4r+} and T_{6r+} phages comes from the medium surrounding the infected bacterium. Similar results have been obtained by Labaw⁹ for T_{2r} and T_{2r+} .

With T_{6r+} , similar values to those observed in phosphorus transfer are obtained when N^{15} is used as the label, and we have found that 80 per cent of the N of T_{6r+} comes from the constituents of the medium.¹⁰ This is to be contrasted with phages T_1 , T_3 and T_7 , in which Labaw⁹ finds that 39 per cent, 17 per cent and 15 per cent, respectively, of the viral P is derived from the medium and that the bulk of the viral P comes from the bacterial host. The data from T_5 are anomalous, but Labaw asserts that a re-examination of the physical properties of this virus indicates that it is of the same size, or slightly smaller, than T_2 , and suggests that it belongs in this group, although it is serologically unrelated to these phages. One may ask, then, whether

TABLE 1
SIZE, SHAPE, AND MOLECULAR CONSTANTS OF SOME PURIFIED BACTERIOPHAGES*

Type	Electron microscope		Ultracentrifuge		Diffusion		Specific volume	Infectivity		Particle weight	
	head diam	tail	S_{20}	d_{eq}^\dagger	D_{20}	d_{eq}^\dagger				Ms, D	Biol.
	m μ	m μ	10^{-13} sec	m μ	10^{-7} cm ² sec ⁻¹	m μ	ml/g	g N / phage	m μ	10^6	10^6
Cotiphages†	50	120×10									
	90	170×15									
	60×80	100×20	1000 700	59 50			0.66	1.3×10^{-16}	107		584
	60×80	100×20			0.80	53					
	60×80	100×20	1050 825	61 54	0.45	94	(0.66)	1×10^{-16}	98	167	460
Staph. phage	45	none			1.19	36				31	
	45	none	480 650	43 77	0.18	235	0.68 0.83	0.5×10^{-16} 1×10^{-16}	80 93	530	246 300

* For references, see the text.

† d_{eq} —The apparent spherical diameter, i.e., the equivalent diameter of an unhydrated sphere of the same mass and volume.

‡ Scale: 1 cm = 150 m μ .

there is any reason for this striking difference between the T₂ groups of phages and a phage such as T₇.

The bacterial cell, of course, contains more than enough material for the synthesis of the virus if we think in terms of N, C and P; but this is not the case if we consider the chemical form in which these elements occur. Un-

TABLE 2
DISTRIBUTION OF P IN *E. COLI* FROM BROTH AND SYNTHETIC MEDIA

Type of P	Broth bacteria*	Synthetic medium bacteria†
	per cent	per cent
Acid soluble.....	20.7 ± 2.4	24.6 ± 1.4
Alcohol soluble.....	11.1 ± 0.9	15.7 ± 5.0
RNA†.....	48.5 ± 1.9	38.4 ± 4.8
DNA†.....	13.1 ± 0.1	22.6 ± 3.5
Phosphoprotein.....	5.2 ± 3.0	4.4 ± 0.6
RNA P/DNA P.....	3.5 ± 0.1	1.8 ± 0.4

* Average of 5 analyses.

† Average of 4 analyses.

‡ RNA denotes ribonucleic acid; DNA, deoxyribonucleic acid.

TABLE 3
DISTRIBUTION OF P. IN *E. COLI* BACTERIOPHAGE T₆ FROM BROTH CULTURES^a

Type of P	Preparation Number								Average	Broth ^c bacterio- phage T ₂		
	XVI ^b		XVI ^c		X		III				II	
	per cent	Pn/P	per cent		Pn/P	per cent	Pn/P	per cent		per cent		per cent
Acid-soluble total P	2.65		3.6		7.0		2.0		2.9		1.0	3.2 ± 2.0
(inorganic)	(0.6)						(0.6)				(0.2)	
Alcohol soluble	1.2		1.1		1.0		2.0		2.6		1.2	1.5 ± 0.2
DNA ^d	83.8	0.94	84.2		81.5	0.99	82.0	0.92	82.0		88.7	83.5 ± 2.5
RNA ^d	7.1	0.83			5.6	0.80	8.9		6.8		5.8	7.0 ± 1.1
Residue	5.1		11.0		5.0		4.9		5.7		3.4	4.8 ± 0.2
Infectivity (gm. of N per phage)	10 ^{-16.0}		10 ^{-15.8}		10 ^{-15.7}		10 ^{-15.7}		10 ^{-15.8}			10 ^{-15.8}
P per phage (× 10 ¹¹)	3.3		—		4.8		—		3.4		4.2	3.9
												—

^a Values for each fraction represent the per cent of total phage P. Figures for inorganic P in parentheses are included in total acid soluble P. Italicized figures give the ratio of DNA or RNA P (Pn) found in that fraction by the cysteine or orcinol reactions to P actually found for the fraction.

^b Homogeneous in electrophoresis.

^c Same as above but incubated with trypsin for 30 hours and repurified.

^d DNA denotes deoxyribonucleic acid; RNA; ribonucleic acid.

^e Data calculated from (1).

^f Includes inorganic P.

fortunately, we cannot give a complete analytical account of the chemical composition of the bacterial cell, and it is necessary to resort to arbitrary chemical classification. By use of well-known chemical procedures, we can subdivide the N and the P of the bacterial cell into four classes: the acid-soluble material, which consists primarily of low molecular weight nitrogenous and phosphorus compounds; the alcohol-soluble fraction, which contains lipid material; and the protein and the nucleic acid fractions, the last being

divided into RNA and DNA subdivisions. It is clear, of course, that such chemical distinctions may group together compounds of quite unlike physiological importance, and our conclusions must always be examined from this point. Analytical data for the *E. coli* bacterial cell and the bacteriophage T_6 are shown in TABLES 2 and 3.

There is one point in particular that seems pertinent to the observation that the tailed phages derive the bulk of their protoplasm (if one might use the word) from the medium, whereas a spherical phage, such as T_7 , requires little, if any, contribution of outside material. This lies in the relation between the DNA content of the bacterial cell and the amount of DNA which appears in the viral progeny. In the case of T_{2r+} , T_{4r+} and T_{6r+} , there is not enough host DNA to account for that which is present in the viral progeny and, with these phages, the DNA content of the viral progeny may exceed 2 or 3 times the DNA content of the bacterial host. It is clear,

TABLE 4
ORIGIN OF VIRUS NITROGEN
GROWTH OF BACTERIOPHAGE T_{6r+} ON *E. COLI*

	Experiment I <i>N</i> ¹⁵ labeled medium unlabeled bacteria		Experiment II <i>N</i> ¹⁵ labeled bacteria unlabeled medium	
	Atom % excess	% of N derived from medium	Atom % excess	% of N derived from bacteria
Bacteria, N.....	0.00	—	9.73	—
Medium, N.....	10.1	—	0.00	—
Phage, total N.....	8.19	81.1	2.08	21.4
Phage, nucleic acid N.....	6.73	66.7	—	27.7
Phage, protein N.....	9.21	91.2	.76	7.82
Debris, N.....	2.96	29.3	6.7	69.0

therefore, that a synthesis of viral DNA from some other source must occur. On the other hand, the characteristics of the T_3 and T_7 systems are such that host DNA is sufficient to account for virus DNA and a synthesis from other sources is not necessary.

Before considering whether there is an actual transfer of bacterial DNA to virus, it might be asked whether other components of the bacterial cell can serve as sources of viral DNA. Most of our evidence concerning this point comes from work with the T_{6r+} strain of phage so far as the acid soluble material of the bacteria is concerned, experiments of Putnam and Kozloff¹¹, and of Siddiqi¹² indicate that no major contribution from this fraction occurs. Cohen¹³ has shown that the metabolism of RNA and phospholipid in the infected cell comes to a stop, and these materials cannot participate, therefore, in the manufacture of viral DNA. Finally, Dr. Siddiqi has obtained evidence that bacterial protein is not a major source for viral DNA. We are left finally with the conclusion that in the case of T_{6r+} , and presumably the other phages of this group, the major portion of

viral DNA must be derived from the DNA of the bacterial cell, together with DNA synthesized from the simple constituents of the medium. The participation of constituents of the medium in viral synthesis is demonstrated by experiments, in which, by labeling the N and P constituents of the medium with isotopes, one shows that these materials appear in large quantities in the viral progeny (TABLE 4). So far as DNA transfer is concerned, the most conclusive direct evidence in this respect derives from experiments by Dr. Koch¹⁴, in which only the adenine and guanine of the bacterial nucleic acids were labeled with radioactive C¹⁴. When such specifically labeled cells are infected with T₆r+, the liberated virus isolated, and the purines separated by chromatographic procedures and analyzed, one finds C¹⁴ present only in the adenine and guanine of the virus (TABLE 5). If we compare the results of these experiments with others using a bacterial host cell in which all of the N components have been labeled with N¹⁵, we find further

TABLE 5

TRANSFER AND INTERCONVERSION OF PURINES OF THE HOST DURING MULTIPLICATION OF *E. COLI* BACTERIOPHAGE T₆

Experiment No.	Isotope in purine	Relative specific activity of bacterial DNA		Yield	Phage DNA purine / Host DNA purine × 100		Ratio of last two columns
		Guanine	Adenine		Guanine	Adenine	
M ₁	C ¹⁴	100	100		40		
L ₁	N ¹⁵	100	100	287	21.8	14.0	1.56
L ₂	N ¹⁵	100	100	117	18.8	14.8	1.27
K ₁	C ¹⁴	90	100*	247	20.0	14.0	1.43
K ₂	C ¹⁴	284	100*	200	29.0	21.0	1.43
K ₇	C ¹⁴	41	100*	90	72.8	34.4	2.12
K ₈	C ¹⁴	42.8	100*	68	53.4	28.7	1.86

* In these experiments values are expressed relative to DNA adenine as 100.

support for the transfer of bacterial DNA to virus. Analyses of the virus obtained from such a host shows that the percentage of host isotopic N present in viral DNA is about the same as that indicated by the C¹⁴ experiments, *i.e.*, the magnitude of the transfer as measured by N¹⁵ and by the C¹⁴ labeled purines is the same. Moreover, N¹⁵ is found in the same concentration in both the purines and pyrimidines of viral nucleic acid; this also being observed in the particular case of the pyrimidine base thymine. This latter point is of especial interest since thymine occurs only in the DNA fraction of the bacterial cell and it is clear that not only purines and pyrimidines are transferred intact from host to virus, but that they must originate (at least in part) from the DNA fraction of the bacterial host.

These experiments involved T₆r+ and one may inquire whether evidence exists for a similar transfer of bacterial DNA to virus in the case of the small spherical phages such as T₇. Some recent experiments with this phage, acting on bacteria labeled with P³² and N¹⁵, are pertinent.¹⁵ In mass cultures on synthetic medium, total lysis occurs with T₇ in 40-60 minutes.

Since the specific activity of the acid-soluble P of the bacterial cell changes rapidly when labeled bacteria are suspended in unlabeled medium, the experiments were carried out so as to measure the specific activity of the various fractions of bacterial phosphorus at zero time, *i.e.*, at the moment of suspension in the unlabeled medium and at intervals prior to lysis. TABLE 6 summarizes an experiment of this sort.

It is evident that the specific activity of the virus P was invariably greater than that of the bacterial acid-soluble phosphorus prior to lysis, thus excluding the possibility that much or all of the viral phosphorus (which is, of course, nucleic acid P) originated in the low molecular weight compounds of the acid-soluble phosphorus fraction. Moreover, the specific activity of the viral phosphorus was 59 per cent that of the remaining phosphorus fractions of the host, indicating that all, or nearly all, of the viral phosphorus came

TABLE 6
ORIGIN OF THE P AND N OF *E. COLI* BACTERIOPHAGE T₇ (#66)

	Initial bacteria	Isotopic activity				
		Bacteria at 10 min or final virus				
		Whole	Acid sol	RNA	DNA	Protein
<i>P</i> ³² — c.p.m./γ <i>P</i>						
Virus.....	778	440	115	435	475	355
Bacteria.....		—	332	505	540	500
Ratio.....					0.88	0.71
<i>N</i> ¹⁵ — atom % excess						
Virus.....	9.90	5.74	1.85	3.62	6.53	3.75
Bacteria.....		8.35	5.72	8.14	8.15	8.33
Ratio.....		0.69			0.80	0.45

from host nucleic acid (since bacterial phospholipid phosphorus can be excluded on other grounds). In marked contrast to T₆, all, or nearly all, of the viral phosphorus is derived from the bacterial host and there is minimal utilization of medium P for viral DNA synthesis. The nitrogen figures confirm this and also demonstrate a larger transfer of isotope from host to viral DNA than from host to viral protein. With insufficient host DNA to account for viral DNA, extensive utilization of the components of the medium takes place in T₆. With T₇ in which there is enough host DNA to supply the total DNA requirement of the viral progeny, there is little utilization of the medium. This certainly suggests that the transfer of bacterial DNA to viral DNA plays an important role in the process of coliphage replication.

This leads to the question as to whether there is anything specific or unique about this contribution of bacterial DNA to viral progeny. As I have

already pointed out, since there are marked contrasts in the composition of the nucleic acid from host and from virus, it is clear that the transfer must involve fundamental rearrangements and alterations. Indeed, experiments of Koch and of Kozloff and Putnam indicate this to be the case. Apart from this, it is still uncertain whether we are concerned here with a type of specific and obligate transfer of host DNA to viral progeny, or whether the appearance of particular molecules of bacterial DNA in the viral progeny indicates merely that host DNA serves as a convenient and readily available chemical "bone-pile" from which materials can be readily and conveniently abstracted for the replication of the viral particle. Such a view is supported by the recent experiments of Weed and Cohen,¹⁶ who have infected bacteria, in which the pyrimidines of the nucleic acid were labeled with C¹⁴, with T₆r+ and T₆r (the slow "lysis-inhibited" strain and its one-step mutant, rapid lysing strain). They analyzed the isotope progeny after normal lysis as well as phage obtained by premature lysis of the infected cells with NaCN. It was found that the thymidylic and desoxycytidylic acids of the virus derived from the early lysing T₆r cells and the prematurely lysed T₆r+ cells, contained about four times the isotopic concentration of the pyrimidines present in the virus derived from the cells infected with T₆r+ when lysis was delayed and the yield of virus greater. Weed and Cohen concluded, therefore, that the virus particles synthesized in the earlier stages of viral reproduction contain the whole of the pyrimidines derived from the host and that the latter particles are composed from material derived from the medium. These experiments, while indicating that pyrimidines, like purines, are transferred intact from host to virus, and also that host DNA is a precursor of viral DNA, disaffirm the concept of a specific material host contribution to each virus particle. Dr. Kozloff, in our laboratory, has carried out similar preliminary experiments, using bacterial cells uniformly labeled with N¹⁵. He has infected such cells with T₆r+ and compared the percentage of isotope in the virus, obtained by premature lysis with sodium cyanide, with that of virus from normal lysis, also to find that the particles formed during early lysis contain more of the host isotope than do those produced at the end, although the differences are less pronounced than in the experiments of Weed and Cohen.

These data are difficult to reconcile with those from earlier experiments with T₆r+, in which, as lysis proceeded, successive batches of phage were harvested, purified, and examined for isotope content. As shown in TABLE 7, the isotopic content of the DNA of phage liberated at different times during lysis remains relatively constant. The level of isotope incorporation varies from experiment to experiment, but in a single experiment, the level remains the same, irrespective of the time of release of the phage particles. This suggested to us the important possibility that the transfer of bacterial DNA to viral progeny involves a donation of individual fragments, possibly identical, of bacterial DNA to each virus particle synthesized. The mechanism of virus replication would be such, then, that each virus particle receives a fragment of the DNA of the host from which it is derived.

One might suggest the possibility of explaining the results of such experi-

ments on other grounds. If we assumed that in our suspension of infected cells bacteriophage synthesis had occurred in each cell at the same rate and to the same extent, and that lysis then occurred independently, liberating preformed phage at varying intervals, one would expect that the percentage of incorporation of host DNA nitrogen would remain the same for phage released over the whole course of the lysis period. However, opposing such an interpretation, is an argument that can be derived from the same experiment. If we compare the figures for isotope contribution to viral DNA with those for isotope contribution to viral protein, we see that the latter fall off rapidly with increasing time. If we were concerned with a process by which simultaneously synthesized virus particles were gradually released over a period of time, we would expect to find the protein nitrogen exhibiting the same constancy with regard to host isotope contribution as does the DNA. I have mentioned Cohen's demonstration that viral protein synthesis apparently precedes that of viral DNA. It hardly seems likely

TABLE 7
KINETICS OF CONTRIBUTION OF BACTERIAL N TO BACTERIOPHAGE

	<i>Per cent N derived from host*</i>					
	<i>Experiment 8</i>			<i>Experiment 11</i>		
Incubation time, <i>hrs</i>	5.5	7	24	3	5	24
Total phage N	28.9	26.9	25.9	38.3	31.1	26.3
Phage nucleic acid N	37.1	38.1	37.9	42.7	43.2	38.2
Phage protein N	17.6	17.4	11.0	26.6	12.7	10.5

Atom % excess N¹⁵ in virus N

* Atom % excess in bacteria prior to infection $\times 100$.

that bacteriophage protein, once synthesized and awaiting release by lysis, would exchange with the nitrogen of the cell or medium while the nucleic acid component of the phage remained inert.

Some experiments of Labaw are also pertinent to this question.⁹ FIGURE 1 is from a recent paper by this investigator. He finds the host contribution of phosphorus to viral progeny roughly the same for all of the coliphages examined; that is, the absolute amount of virus phosphorus (that is, DNA) derived from the host in the case of T₆r+ is approximately that in the case of T₇, although in the one case, the viral DNA is supplemented by a considerable amount of material synthesized from the medium, in the other, practically all of the viral DNA comes from the host.

If one assumes that the major source of the P of viral DNA is the P of bacterial DNA, it appears that the only way to explain all of these results would be to assume that a certain proportion of bacterial DNA is available for the synthesis of virus DNA, and that this same portion is available for the synthesis of any phage strain to which the bacterial host is susceptible. Once this limited quantity of bacterial DNA is used up, further synthesis of viral protoplasm uses the materials of the medium. One might speculate that such a special fraction of bacterial DNA occurs in a form in which its

component purines and pyrimidines, possibly its nucleosides and nucleotides, could be used for the synthesis of viral nucleic acid although none of its specific structural qualities are preserved after its incorporation into viral DNA. I have already pointed out that our chemical procedures of fractionation may include materials of diverse physiological function under a

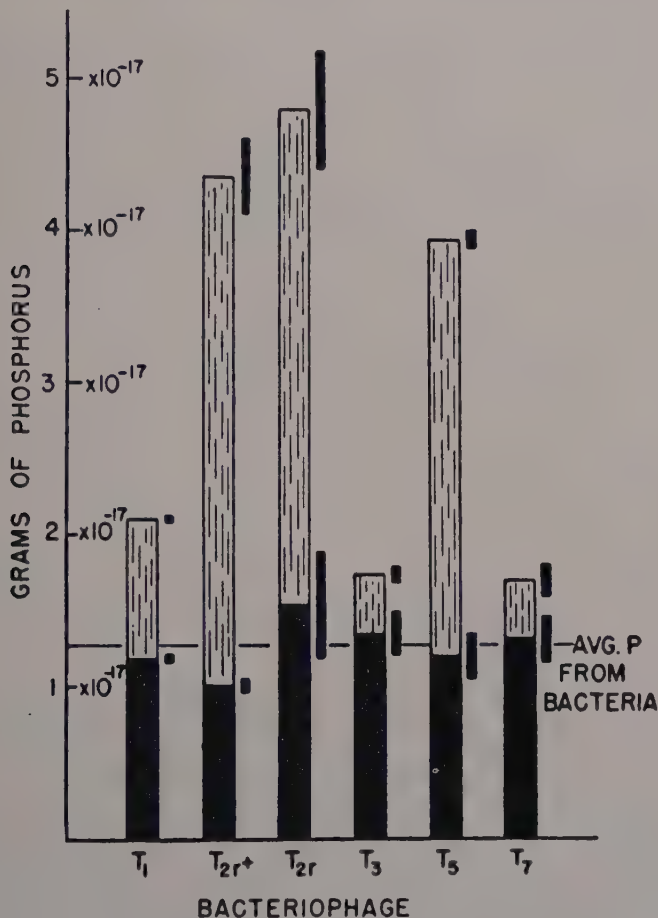


FIGURE 1. A bar graph of the total phosphorus per lytic unit with the virus phosphorus from the host bacteria superimposed. The narrow bars to the right of the main ones represent the mean deviation in the average values plotted.

common chemical label. If this should be the case, bacterial DNA might then include a fraction unavailable for virus synthesis, plus a fraction which, because of either its particular chemical or physical state, can be converted (possibly through the reversal of the reactions by which it is synthesized) into a pool of component materials from which viral DNA is synthesized. If this should be the case (let me emphasize that it is speculation at the moment) such a fraction of host DNA must be characterized by including

the whole of the adenine content of the bacterial DNA, since Dr. Koch has found that the whole of the host DNA adenine appears in the viral DNA.¹⁴

A similar speculation might also be made for bacterial protein, although in this case the amount of bacterial protein available for the synthetic pool would have to be of much smaller magnitude than in the case of DNA in order to explain the rapid rate at which the bacterial protein isotope is diluted out in the process of virus replication.¹⁰

Let us turn finally to the third question (See page 909), and to our information regarding the fate of the infecting particle which initiates the process of viral replication. Omitting from review a great deal of important and interesting work about the mechanism of the absorption process, perhaps the most striking conclusion arising from this phase of viral research is that the intracellular process of viral replication is apparently initiated by only a fragment of the original particle. This explains the fact that virus cannot be detected in the infected host until shortly before lysis occurs.

TABLE 8
DISTRIBUTION OF ISOTOPE AFTER MULTIPLE INFECTION OF *E. COLI* WITH BACTERIOPHAGE T_6r^+ CONTAINING N^{15} *

Material	Per cent of parent T_6 N^{15}
N^{15} split off after 5' adsorption.....	25
Crude lysate.....	75
Bacterial debris.....	3.5
High speed supernatant.....	19.3
T_6 progeny.....	18.1

* Multiplicity of infection was 4.8 T_6 per bacterium. The parent phage contained 62 atom per cent excess N^{15} .

Experiments on the fate of the infecting particle can be performed conveniently by marking the virus with isotopic N, P or C. Soon after the crystallization of the tobacco mosaic viruses, the first experiments of this type were carried out by Stanley, who used virus marked with radioactive P^{32} . He found that the P^{32} of the parent virus appeared in the viral progeny and in various phosphorus-containing, non-viral fractions of the diseased plant. Experiments with P^{32} marked bacteriophage were first performed by Kozloff and Putnam and more recent experiments have been carried out by Kozloff with T_6r^+ labeled with N^{15} . TABLE 8 shows the distribution of isotopic N^{15} in materials fractionated from the lysates obtained after infection of *E. coli* with N^{15} labeled bacteriophage T_6 . Most of the parent viral N appears in the non-viral fraction: the viral progeny contain only 18 per cent of the original N of the parent particle. Another experiment in which the appearance of non-viral N^{15} was followed until bacterial lysis was complete is summarized in FIGURE 2. Here, approximately 35-40 per cent of the N of the parent virus is split off and appears free of the infected cell within a short interval after adsorption. After this initial release of non-viral N^{15} , no more isotope appears in the medium until lysis begins. At the end

of lysis, 60–80 per cent of the original viral N occurs in the non-sedimentable fraction, *i.e.*, is present as substances of low molecular weight which cannot be sedimented by centrifugal forces capable of throwing down the original virus particle. Examination of the chemical nature of the non-sedimentable material indicates that appreciable quantities of the original viral N appear in the supernatant as ammonia or as amide nitrogen, and that less than 20 per cent of the supernatant N can be precipitated as protein. Experiments using virus labeled with P^{32} and with virus containing both N^{15} and P^{32} lead to the same conclusion.

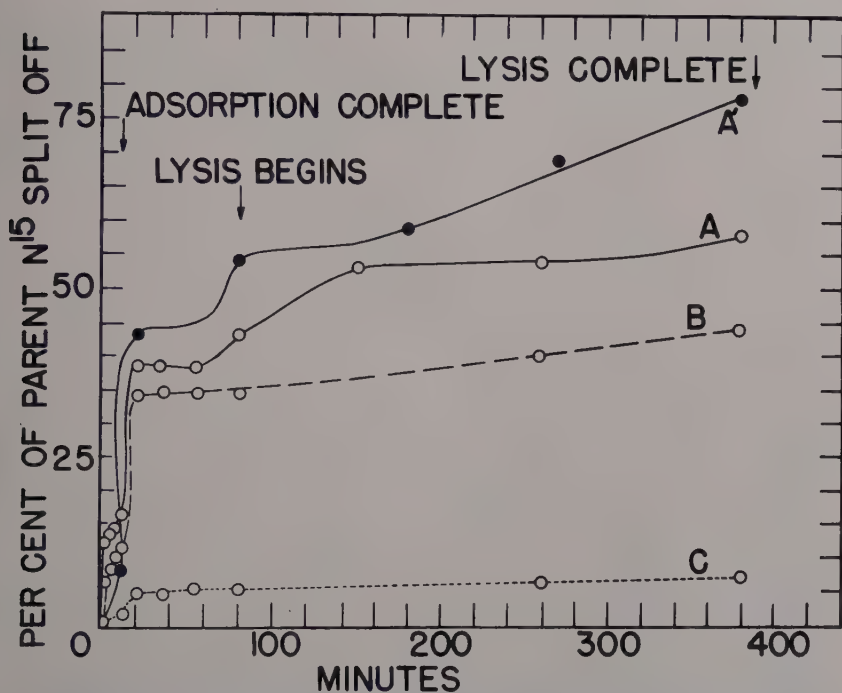


FIGURE 2. The breakdown of N^{15} labeled parent Tar^+ bacteriophage during virus growth. Experiment N^{15} III K_1 (○) Curve A — non-viral N^{15} which could not be sedimented at $3500\times g$; Curve B — non-viral N^{15} which could not be sedimented at $18,000\times g$; Curve C — N^{15} which could not be distilled over by the addition of alkali. Experiment N^{15} IV (●) Curve A' — non-viral N^{15} which could not be sedimented at $3000\times g$.

Lesley, French, and Graham, at Toronto¹⁸⁻²⁰ have presented evidence that this initial fission of the phage particle may be of two types: (1) of smaller magnitude associated with the absorption and penetration of the bacterial cell by the initial infecting particles; and (2) much greater in magnitude, caused by the interaction of the infected bacterial cell with additional virus particles. In any event, one observes the transfer of about one-third of the labeled materials (N, P) of the virus particle to the viral progeny, usually with a somewhat larger contribution coming from the parent nucleic acid than from the parent protein. This, however, does not represent the transmission of any specific molecular entity.

Recently, Hershey, Kamen, Gest, and Kennedy²¹ determined, in an ingenious manner, the distribution of parent virus P among the many phage produced. They measured the mortality rate of bacteriophage T₄ produced by infection of bacteria with unlabeled parent T₄, in a medium containing concentrations of P³² sufficient to cause death of the new phage by the disintegration of the assimilated P³². Then, in their experiment, the parent T₄ is labeled with the normal variety of Phosphorus P³¹. They concluded that, since the mortality rate of the particle phages produced in the experiment was identical for 99.9 per cent of the phage, the unlabeled parent material (which should increase the survival time) was dispersed equally among all the progeny and was not concentrated in only a few of them. This equal distribution of parent material prompted these workers to suggest that the parent contribution may consist of essential structures which are conserved. This suggestion, however, is not in accord with recent work. Dr. Kozloff has found that labeled particles which have been inactivated by ultraviolet

TABLE 9
PROCEDURE FOR DETERMINING EFFECTS OF RADIATION ON THE CONTRIBUTION OF PARENT MATERIAL TO VIRUS PROGENY

Purified N ¹⁵ Labeled Parent T ₆	
Ultraviolet light 99.85% inactivated 6.4 hits	X-rays, 300,000 99.84% inactivated 6.4 hits
+	+
Equal number of live unlabeled T ₆	Equal number of live unlabeled T ₆
↓	↓
Infect unlabeled <i>E. coli</i> in unlabeled medium	Infect unlabeled <i>E. coli</i> in unlabeled medium
↓	↓
T ₆ progeny containing N ¹⁵	T ₆ progeny containing N ¹⁵

light or by X-rays, contributed just about as much of their labeled components to viral progeny as occurs with active virus¹⁵ (TABLES 9 and 10).

In another experiment, a large culture of bacterial cells were infected with unlabeled T₇ bacteriophage and P³²-labeled T₆ bacteriophage. There was growth of both viruses, but presumably in different host cells since it has been shown that these two viruses do not multiply in the same cell. TABLE 11 shows the radioactivity and virus content of the successive purified virus concentrates obtained from this mixed lysate. Removal of most of the T₆ by selective adsorption with a bacterial mutant (B/3, 4, 7) made it possible to calculate the amount of radioactivity in both the T₇ and the T₆ by solving simultaneous equations relating the titers to the radioactivity. There can be little doubt that there is P³² in both the T₇ and T₆ progeny. The distribution of the P³² in the lysate shows that while the T₆ progeny contained 37 per cent of the P³², 4.6 per cent appeared in the T₇ progeny (TABLE 12). In view of the fact that T₆ and T₇ do not multiply in the same cell, and that therefore there cannot be genetic interaction in the usual sense between these two phages, the appearance of P³² from the T₆ parent in the T₇ progeny is

probably due to the use of fragments of the DNA of the T₆ for the synthesis of the T₇ nucleic acid. It is clear that if material can be transferred in a non-specific manner between unrelated phages a similar process could occur with related phages.

The relatively equal distribution of parent P³¹ observed by Hershey and his associates is apparently not of crucial importance, and may be attributed to the net effect of the numerous chemical reactions occurring inside the cell (such as the rate of breakdown of parent nucleoprotein and the rate of synthesis of the progeny nucleoprotein) and not to any specific genetic mechanism.

It cannot be claimed with complete assurance that absolutely no genetic units are contributed by the parent to the offspring. A small contribution of

TABLE 10
EFFECT OF ULTRAVIOLET AND X-RAY IRRADIATION ON THE CONTRIBUTION OF PARENT N¹⁵ TO VIRUS PROGENY

<i>Expt.</i>	<i>Irradiation</i>		<i>Multiplicity of infection</i>	<i>Parent N¹⁵ contributed to progeny T₆</i>
	<i>type</i>	<i>dose in hrs</i>		
—	None	—	2.5 × 12	4.5–18
N ¹⁵ III	X-ray	6.25*	8.0	14.9
N ¹⁵ VB	UV	2.1	2.5	8.0
N ¹⁵ VC	UV	2.1	4.7	8.8
N ¹⁵ VA	UV	2.1*	4.7	10.3
N ¹⁵ III	UV	6.4*	3.5	13.2
N ¹⁵ IVA	UV	8.2*	5.2	10.9
N ¹⁵ IVB	UV	16.4*	5.2	13.1
N ¹⁵ VD	UV	25.3*	5.0	8.6
N ¹⁵ IVC	UV	28.7*	5.2	9.1
N ¹⁵ IVD	UV	49.0*	5.1	7.0
N ¹⁵ VE	UV	63.0*	4.8	10.6
N ¹⁵ VF	UV	210.0*	4.9	4.8
N ¹⁵ P ³² III	UV	250.0*	8.0	10.0

* In these experiments the N¹⁵ labeled phage was supplemented with live unlabeled phage.

genetic units overlaid by the non-specific transmission of isotope material would be hard to prove or disprove by these techniques. Any material genetic contribution, however, must be very small indeed, since the total and mainly non-specific contribution of parent virus N and P (assuming 150 progeny per parent particle and the transfer of 15 per cent of the parent material) amounts to only 0.1 per cent of the total nucleoprotein of the progeny.

The biochemical facts that we have considered fit into a picture of viral reproduction that does not differ drastically from deductions previously made by other workers. Since, with the coliphages at least, the absorption of the infecting virus particle is associated with its fragmentation, it appears that the actual process of viral synthesis is not initiated by the virus particle as we know it, but only by a portion of it. Further, since the actual materials of this surviving fragment are handed on to the progeny in a random fashion, the role of the effective fraction of the virus appears to initiate a

distortion or transformation of the normal metabolic activities of the cell into those associated with the manufacture of new virus particles. Having done this, portions of the particle are used non-specifically in the synthetic process. Aside from the fact that large amounts of bacterial DNA are used for the synthesis of viral progeny (this may mean simply that such material is easily available), there is no evidence that the various constituents of the host cell are specifically involved in viral synthesis. Once again, it would appear that it is a case of the normal machinery of the cell being converted

TABLE 11
P³² RADIOACTIVITY OF PHAGE CONCENTRATES FROM MIXED T₇, T₆ LYSATE^a

Concentrate	T ₇ per ml	T ₆ per ml	c.p.m./ml	T ₇ cts. (calc)	T ₆ cts. (calc)	T ₇ + T ₆ cts. (calc)
T ₇ ^b	3.8×10^{11}	8×10^{10}	393	122	272	394
T _{7a} ^c	4.6×10^{11}	8×10^{10}	422	147	272	419
T _{7b} ^d	3.7×10^{11}	1.1×10^{10}	168	119	37	156
T _{7c} ^e	5×10^{11}	4×10^9	160	160	14	174
Calculated cts/phage ^e						
c.p.m./T ₇		3.2×10^{-10}				
c.p.m./T ₆		3.4×10^{-9}				

^a All radioactivity was initially in the T₆ which was added 8 minutes after infection with unlabeled T₇.

^b Concentrate obtained after removal of all non-viral material.

^c Recycled T₇ concentrate.

^d Successive concentrates obtained after removal of T₆ by adsorption on *E. coli* strain B/3, 4, 7.

^e Cts/phage calculated by solving the simultaneous equations relating the titers and the amounts of radioactivity.

TABLE 12
DISTRIBUTION OF RADIOACTIVITY AFTER INFECTION WITH UNLABELED T₇ BACTERIOPHAGE AND P³² LABELED T₆ BACTERIOPHAGE

Fraction	c.p.m./phage	Titer of phage	c.p.m./ml	Per cent of cts.
Cts. split off during T ₆ adsorption....	—	—	—	16
Lysate.....	—	—	25.5	84
T ₆ in lysate.....	3.4×10^{-9}	3.3×10^9	11.2*	37
T ₇ in lysate.....	3.2×10^{-10}	4.4×10^9	1.4*	4.6

* Calculated from the titer and the radioactivities determined on the phage concentrates.

to the synthesis of virus particles. Increased knowledge of the biological nature of the virus particle may afford further insight into the process of virus replication. In this respect, the recent work of Lwoff and his collaborators on lysogenic strains of bacteria seems to be of the greatest importance.^{22, 23} In such strains, the French workers have been able to induce, by treatment with ultraviolet, X-rays, or a number of chemical substances, the appearance of phage, *i.e.*, we have for the first time a situation in which virus production is caused by a chemical or physical agent acting on a cell apparently free of virus. Here, also, the nucleic acid of the lysogenic bacterium seems involved, and it is clear that a more detailed knowledge of the nucleic

acid metabolism of the bacterial cell will prove to be of the greatest importance in understanding the nature of virus reproduction in bacterial hosts.

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FACTORS CONCERNED WITH VIRAL PROLIFERATION IN VIVO

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When a virus and a living cell are brought close together, there is no discernible reaction between them in the great majority of instances. However, if the virus and the host cell possess those complementary properties which permit designation of the virus as infectious and the host cell as susceptible, then a series of reactions may occur which leads to an increase in the amount of virus. The reactions which follow infection of a susceptible host cell with a virus have many aspects (Woodruff and Goodpasture, 1930). One of these is the stepwise process which results in the development of new viral particles (Burnet, 1929; Ellis and Delbrück, 1939; Delbrück, 1940a and 1940b; Henle, *et al.* 1947; Ginsberg and Horsfall, 1949a and 1951a). This process is termed viral multiplication or proliferation and is affected in various ways by a large number of factors (Horsfall, 1949). Some of the more prominent factors are: (1) the genetic features of the host cell and the virus; (2) the metabolic state of the host cell; (3) the presence of another virus; and (4) the composition of the extracellular environment. The most evident ways in which such factors act are the augmentation or inhibition of viral proliferation so that either more or less virus develops (Horsfall, 1951).

Before proceeding to a more detailed consideration of factors which, at the cellular level, have an effect upon the multiplication of viruses, another aspect of animal virus infections deserves emphasis. Viruses which induce infections in animal species do not usually come into contact with host cells susceptible to infection immediately after gaining entry to the body. In most instances, the infectious agent is transported from the portal of entry to the tissue that contains susceptible cells. In no case is it known that all types of cells are equally susceptible to infection. In many instances, susceptibility is restricted to cells of a single type; in some, to cells present only in a single tissue. During the period of transport to susceptible cells, the naked viral particle is in a highly vulnerable condition and can be prevented readily from reaching cells it can infect.

The most specific and probably the most effective substances which can act at this stage are antibodies directed against the agent. Specific antiviral antibodies develop as a result of contact with the agent. Usually, though not always, they are a reflection of immunity and are found not only in the blood, but also in the tissue fluids, as well as in the secretions of the mucous membranes (Francis, *et al.* 1943). Much evidence indicates that the interaction between antibody and viral particle prevents adsorption of the virus by susceptible cells and thus the process of multiplication does not begin (Delbrück, 1945a; Volkert and Horsfall, 1947). In contrast to the striking effectiveness of antibodies in preventing infection of the cell is their marked lack of effect upon viral proliferation once the virus has pene-

trated the cell (Delbrück, 1945a; Ginsberg and Horsfall, 1951b; Taylor, 1941; Henle and Henle, 1949). Large amounts of antibody introduced after this stage have little or no effect upon multiplication. When viral particles can migrate from one cell to the next without coming into contact with extracellular fluid, as through intercell bridges, the presence of antibodies may have no demonstrable effect upon successive cycles of multiplication which then can proceed uninhibited.

Substances other than antibodies are capable of reacting with viruses and also are present in the blood as well as in mucous secretions. None of these components has been identified adequately. Some are as labile as hemolytic complement (Douglas and Smith, 1930; Ginsberg and Horsfall, 1949b); others are much more stable (Casals and Olitsky, 1947; Francis, *et al.* 1947; Mandel, 1951; Burnet and McCrea, 1946), but certain of them can inactivate a number of viruses directly and thereby prevent infection of susceptible cells. There is, as yet, no means for predicting whether substances which interact with viruses, under the conditions of *in vitro* tests, will also cause inactivation of viral infectivity. Certain highly reactive components, such as muco-proteins (Tamm and Horsfall, 1952), which react with influenza or mumps viruses have little or no effect upon infectivity (Hardy and Horsfall, 1948; Burnet, 1948), while others, such as a heat labile component of normal serum (Ginsberg and Horsfall, 1949b), destroy the infectivity of these and other viral agents.

The genetic features of both the host cell and the virus are beyond the range of direct experimental control but are within the realm of manipulability. Susceptibility of the host species to infection with a given virus is clearly an inherited character. This is most evident with bacterial host cells which give rise to mutants that are susceptible to one but not to the other of two viruses (Luria and Delbrück, 1943). With animal hosts, particularly the mouse, suggestive results have been obtained and there are indications that variation in susceptibility is subject to genetic control (Webster, 1937; Sabin, 1952). Although the degree of susceptibility, like pathogenicity, is not completely correlated with the extent of viral multiplication, there is a relation between the two variables.

Viral mutants appear to emerge continuously, though in low frequency, and, in the case of animal viruses, are vastly more arresting than are genetic alterations in the host. By appropriate management of the environment, it is possible to select mutant viruses that may have predictable properties (Ginsberg and Horsfall, 1949a; Björkman and Horsfall, 1948; Archetti and Horsfall, 1950). Such mutant agents are often capable of multiplication in host cells which are not able to support proliferation of the parent or wild type virus. Mutants of influenza (Stuart-Harris, 1939) and dengue viruses (Schlesinger and Sabin, 1945), which multiply in the mouse brain; and mumps (Habel, 1945) and measles (Rake and Shaffer, 1940) viruses, which proliferate in the chorio-allantoic membrane of the chick embryo, have been obtained. Markedly enhanced or strikingly diminished pathogenicity may characterize some viral mutants and, in general, there is a positive correlation between the degree of pathogenicity and the rate or extent of multiplica-

tion (Davenport and Francis, 1951; Wang, 1948). The selection of mutant viruses of diminished pathogenicity which lead to the development of immunity rather than to the induction of manifest disease has made possible the achievement of successful prophylaxis against smallpox, yellow fever (Lloyd, *et al.*, 1936), rinderpest (Jenkins and Shope, 1946), and rabies (Koprowski and Cox, 1948), and has raised the possibility of eventual control of measles (Maris, *et al.*, 1943) and mumps (Enders, *et al.*, 1946). Like microbial species, viruses can yield mutants that circumvent the effects of chemical substances which interrupt multiplication of the parent agent. Mumps virus is a case in point; a resistant variant capable of unrestricted proliferation in the presence of relatively large quantities of *K. pneumoniae* capsular polysaccharide has been obtained from this agent (Ginsberg and Horsfall, 1949a).

Even the antigenic character of some viruses is subject to alteration as a result of selective procedures during multiplication. This has been most clearly demonstrated with influenza viruses by means of serial passage in the mouse lung (Hirst, 1947), selection at limiting infective dilution (Isaacs and Edney, 1950a), and incomplete neutralization with immune serum *in ovo* (Archetti and Horsfall, 1950). The progressive alteration in the antigenic composition of influenza viruses associated with epidemics occurring during the last few years (Francis, *et al.*, 1947; Rasmussen, *et al.*, 1948; Taylor, 1949; Hilleman, *et al.*, 1950a and 1950b) can be understood in the light of recent findings. Persons possessing adequate antibodies against some strains of influenza A virus are relatively resistant to infection with antigenically similar strains (Commission on Influenza, 1944; Hirst, *et al.*, 1944) but are susceptible to infection with antigenically different strains (Francis, *et al.*, 1947). Such persons would serve as natural selectors and foster the proliferation of mutant influenza A viruses with new antigenic characters. Continued exposure through natural infection or artificial immunization to a restricted segment of the antigenic potentialities of a given virus thus could lead to the emergence of an agent with a different antigenic composition (Archetti and Horsfall, 1950). The new virus should lead in time to the development of relatively specific immunity against itself and thereby contribute to the selection and predominance of further mutants with dissimilar antigenic characters. If, during this process, a mutant virus appeared which possessed not only an altered antigenic composition, but also possessed increased pathogenicity, or an augmented capacity to multiply, as has been repeatedly observed with influenza virus in mice (Davenport and Francis, 1951; Wang, 1948), the results might be highly unpleasant.

Recent work has shown that certain hormones exert surprising effects upon the process of multiplication of some animal viruses. The most striking results have been obtained with the adrenal steroid, cortisone. When a relatively large amount, one to five milligrams, is injected shortly before influenza or mumps virus is inoculated into the allantoic sac, the quantity of virus obtained on multiplication ranges from 120 to 470 per cent of that found in control embryos (Kilbourne and Horsfall, 1951a). The increased viral proliferation contrasts sharply with the marked stunting

effect of cortisone on embryo growth (Karnofsky, *et al.*, 1950). Infection of mice and hamsters with poliomyelitis virus is enhanced by cortisone (Schwartzman, 1950) and it has been demonstrated that multiplication of this virus also is augmented by the substance (Sabin, 1952). The adult mouse, which normally is almost completely resistant to infection with Coxsackie viruses, is converted by a single injection of 2.5 to 5 milligrams of cortisone into an animal highly susceptible to infection with certain of these agents and supports their multiplication to an extent comparable to that obtained in infant mice (Kilbourne and Horsfall, 1951b). The susceptibility of mice to lethal infection with each of a number of neurotropic viruses is increased by cortisone (Southam and Babcock, 1951). Testosterone and ACTH have been found to affect the extent of multiplication of influenza virus in the mouse lung in a manner which appears to be directly correlated with the effects of these substances on protein metabolism (Kalter, *et al.*, 1951). There is, as yet, no indication of the mechanism that enables steroid hormones to influence viral proliferation in animals nor is it known how many different viruses can be affected by such substances. It appears that the quantity of cortisone given and the duration of its administration may have marked effects upon the results obtained (Kilbourne, 1951).

The capacity of a susceptible host cell to support viral multiplication is, in general, directly related to its metabolic state. With bacterial viruses, a more rapid rate of growth of the bacterial host causes a greater yield of virus per cell (Delbrück, 1940b; Northrop, 1951). Conversely, bacterial cells grown in culture media that fail to support a maximal rate of growth almost invariably yield diminished amounts of virus (Delbrück, 1940b; Northrop, 1951). With animal viruses, there are indications that a similar relation exists between the nutritional state of the host and multiplication of the agent. Reduction of the total caloric intake, as well as restriction in the intake of individual dietary constituents or accessory food factors usually leads to reduction in susceptibility, which has been attributed to a diminished capacity to support viral multiplication (Mirick and Leftwich, 1949). To a degree, the decrease in proliferation of the agent is approximately proportional to the extent of restriction of the nutrient.

Recent studies have raised the possibility that the availability of certain individual amino acids may have a limiting effect upon the multiplication of some viruses. Analogues of tryptophane inhibit the proliferation of bacterial virus T₂ in the host cell *E. coli*, B, and the inhibition is reversed by tryptophane (Cohen and Fowler, 1947). Absence of aspartic acid from the culture medium appears to block the development of a bacterial virus in some strains of *S. muscae* (Price, 1950). Analogues of methionine inhibit the multiplication of influenza A virus in membrane tissue culture and this effect is blocked by *l*-methionine (Ackermann, 1951a). In addition, analogues of methionine cause a decrease in the extent of multiplication of the Lansing strain of poliomyelitis virus in tissue culture (Brown and Ackermann, 1951).

A diet deficient in pyridoxine leads to the formation of considerably less pneumonia virus of mice than develops in control animals given an adequate

diet (Mirick and Leftwich, 1949). Analogues of purines and pyrimidines have been found to diminish the multiplication of vaccinia virus in tissue culture (Thompson, *et al.*, 1950), as well as the multiplication of Russian encephalitis virus, both in tissue culture and in the mouse brain (Moore and Friend, 1951; Friend, 1951). Recently, sodium fluoroacetate has been found to restrain the multiplication of influenza A virus in the mouse lung and the effect has been attributed to blockade of the citric acid cycle of the host cell (Ackermann, 1951b).

The presence of another virus can markedly affect viral multiplication. Inhibition occasioned in this manner is designated as interference (Hoskins, 1935) or, with bacterial viruses, as mutual exclusion (Delbrück, 1945b). Susceptible bacterial cells exposed to two dissimilar viruses support the multiplication of only one of the agents; individual cells never yield both viruses (Delbrück, 1945b; Weigle and Delbrück, 1951). When an interval in time separates the opportunities for infection with the two agents, the agent reaching the cell first is almost invariably the successful member of the pair. If one virus has infected bacterial cells and another is added in considerable excess, multiplication of the first virus is diminished and, on bursting, the infected cells yield less virus than normally (Delbrück, 1945b). This has been termed the depressor effect. Not only are active viruses capable of causing interference, but also those which have been carefully inactivated with ultraviolet light (Luria and Delbrück, 1942).

Closely analogous results have been obtained with many pairs of plant or animal viruses in a wide variety of host species and the interference phenomenon appears now to be of broad applicability in the viral field (Henle, 1950). Interference occurs between viruses with markedly different properties and is not dependent upon an immunological relationship (Ziegler and Horsfall, 1944; Schlesinger, 1952). The phenomenon has been demonstrated most frequently with viruses which infect the same tissue, but it has also been demonstrable when one of a pair of agents showed no evidence of multiplication of infective virus in the tissue inoculated (Vilches and Hirst, 1947). If inactivated agents are employed with animal viruses, it is necessary to give relatively large amounts in order to obtain interference, and the artificial resistance induced is not of long duration (Ziegler, *et al.*, 1944; Henle and Henle, 1944; Isaacs and Edney, 1950b). When infectious virus is used to produce interference, multiplication of a second virus is restrained for a longer period. It appears that the presence of actively multiplying virus leads to a cellular state which prevents proliferation of a second virus, or the same virus when added anew, and that this state may persist as long as multiplication continues. Both in plants and animals, the enduring immunity which follows numerous viral infections appears to be more readily explained on this basis than by the presence of specific antibodies (Horsfall, 1950 and 1951). An analogy may be drawn from recent findings with lysogenic bacteria which are insusceptible to the active virus (Lwoff and Gutmann, 1950) while carrying so-called prophage.

Various mechanisms have been proposed to explain viral interference (Henle, 1950) and two of these have gained considerable support. With certain bacterial viruses of the T series there is evidence that the excluded

virus is rapidly broken up after coming in contact with the infected cell (French, *et al.*, 1951) and probably never enters the cell. With others, there is good evidence that penetration of the infected cell occurs and that interference is an intracellular reaction (Weigle and Delbrück, 1951). In the case of animal viruses, the available evidence supports the concept of competition at the intracellular level (Henle, 1950; Ginsberg and Horsfall, 1949c and 1951c).

It might be thought that if the multiplication of various viruses can be affected, more or less, by such a variety of factors *in vivo*, the development of chemotherapeutic substances effective against viral infections should not be difficult. Extensive investigations have not supported this idea and useful chemotherapeutic substances (Horsfall, 1950) have been discovered only for infections induced with the largest of viruses, the so-called psittacosis-lymphogranuloma group. These substances appear to have little or no effect against other viral agents. The recent intensive search for useful therapeutic substances, which has been conducted in numerous laboratories, appears to have been almost fruitless as far as applicable findings are concerned. Only a few substances are known which are capable of interrupting the multiplication of medium or small size viruses in experimental animals and none has been proven to be effective against similar agents in man.

Among substances which cause inhibition of viral multiplication, only those capable of interrupting the intracellular process appear to merit serious consideration. In only a few instances has it been demonstrated unequivocally that multiplication can be interrupted at the intracellular level. Detailed investigations on the mode and site of action of *K. pneumoniae* capsular polysaccharide in infections with pneumonia virus of mice (Horsfall and McCarty, 1947; Ginsberg and Horsfall, 1951b) or mumps virus (Ginsberg, *et al.*, 1948) have served to exclude other possibilities and leave little doubt that intracellular multiplication of these agents is actually interrupted. This polysaccharide has been shown to be effective during the cell cycle of multiplication if given at any time during the first one-half of the so-called latent or eclipse period (Ginsberg and Horsfall, 1951b). Moreover, the substance has been found to exert a chemotherapeutic effect in infections induced with pneumonia virus of mice even when it is not given until objective evidence of disease has appeared (Ginsberg and Horsfall, 1951b). The results of comprehensive studies with 5-methyl tryptophane (Cohen and Fowler, 1947) or proflavine (Foster, 1948) in infections of *E. coli*, B with bacterial viruses T₂ and T₆ permit similar conclusions. Both substances have been found to interrupt multiplication when they are given as long after infection as one-half of the duration of the eclipse period. In both cases there is evidence indicating that a late stage in the process of multiplication is interrupted and that little or no infective virus is released from infected bacteria. Despite effective interruption of viral multiplication with proflavine, however, there is no evidence of a beneficial chemotherapeutic effect and the infected bacterium undergoes lysis at the expected time (Foster, 1948).

The results of recent studies with methoxinine or ethionine (Ackermann,

1951a) in infections with influenza A virus in membrane tissue culture, as well as those with sodium fluoroacetate (Ackermann, 1951b) in similar infections of the mouse lung, have been interpreted in a similar fashion. Methoxinine appears to interrupt the multiplication of this agent when given during the latent period, and sodium fluoroacetate was found to be effective when administration was withheld until 12 hours after inoculation. Whether any of these compounds can exert a chemotherapeutic effect on experimental infections with influenza A virus in animal hosts remains to be determined.

Each of the substances which have been shown to interrupt multiplication at the intracellular level has been found not to cause inactivation of the virus itself (Cohen and Fowler, 1947; Ackermann, 1951a and 1951b; Ginsberg and Horsfall, 1951b; Ginsberg, *et al.*, 1948; Foster, 1948). Moreover, there is a lack of proportionality between the quantity of the substance used and the extent of the effect upon viral proliferation (Horsfall and McCarty 1947; Ginsberg, *et al.*, 1948). This has been taken as an indication that some intracellular system, limited in quantity, may be involved in the multiplication process (Horsfall, 1951). Reversal of the effect of 5-methyl tryptophane by the addition of tryptophane (Cohen and Fowler, 1947) and reversal of the effect of methioxinine or ethionine by the addition of methionine (Ackermann, 1951a) tend to support this hypothesis.

In the case of *K. pneumoniae* capsular polysaccharide, there is evidence of marked viral specificity and, although a few micrograms of the substance are sufficient to interrupt multiplication of pneumonia virus of mice (Horsfall and McCarty, 1947) or mumps virus (Ginsberg, *et al.*, 1948), much larger quantities have no effect upon the proliferation of influenza or Newcastle disease viruses in the same host species (Horsfall and McCarty, 1947; Ginsberg, *et al.*, 1948). With the five viruses enumerated, there is a striking correlation between the results of interference experiments and experiments on interruption of multiplication by this polysaccharide (Horsfall, 1951; Ginsberg and Horsfall, 1949c and 1951c). In each instance, interference is obtained with like pairs but not with unlike pairs. Viruses affected in a similar manner by the polysaccharide, irrespective of the direction of the effect, give interference and viruses affected in dissimilar ways by the polysaccharide fail to show interference. If the interference phenomenon is to be explained on the basis of competition for a metabolic system at the intracellular level, then it appears probable that interruption of viral multiplication by the polysaccharide can be explained in a similar manner.

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GENETIC, HORMONAL AND AGE FACTORS IN NATURAL RESISTANCE TO CERTAIN VIRUSES

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Natural resistance is used here to designate a refractory state which does not depend on immunologic processes resulting from previous exposure of the host to a particular infectious agent or its component antigens. On this basis, there is evidence that the outcome of certain viral infections can be affected in different ways by the genetic constitution, age and hormonal status of the host. The relationship between the state of the host tissues and the special requirements of individual viruses is so close that the manner in which these factors operate can be illustrated best by an analysis of specific examples.

The plant pathologists were the first to demonstrate by cross-breeding experiments that resistance of various plants to a variety of infectious agents was inborn and depended in some instances on a single pair of genes and in others on multiple pairs. They also showed that inborn resistance to one agent was generally independent of resistance to others. In some instances, susceptibility was inherited as a dominant and, in others, resistance was dominant. The studies of Webster¹ with the viruses of louping ill and St. Louis encephalitis and those of Lynch and Hughes² with the virus of yellow fever in mice provided the first experimental evidence that inherited host factors determined the outcome of mammalian viral infections. Here, too, it became apparent that one could not speak of "virus-susceptible" and "virus-resistant" mice, but only of mice which were susceptible or resistant to a specific virus or group of viruses. The mice which were resistant to the viruses of louping ill, Russian Spring-Summer³ and St. Louis encephalitis were not resistant to the viruses of vesicular stomatitis,⁴ rabies¹ and lymphocytic choriomeningitis.³ A most important contribution to this subject was Webster's demonstration that the inherent resistance and susceptibility of mice to the virus of St. Louis encephalitis was correlated with the level of viral multiplication in the brain, not only *in vivo*⁵ but also in cultures containing minced mouse brain.⁶ Neither Lynch and Hughes nor Webster had strains of mice which were 100 per cent resistant, either originally or after crossing resistant parents. Accordingly, they had difficulty in obtaining satisfactory data for determining the mechanism of inheritance.

I should now like to present some unpublished data of my own which throw additional light on the mechanism of inherited resistance to viral infections. In 1944, I accidentally discovered that the albino mice which were inbred for many years at The Rockefeller Institute at Princeton, N. J. (henceforth called PRI mice) were uniformly resistant to the 17 D strain of yellow fever virus regardless of dosage administered. Early in 1950, I began to breed these mice* in Cincinnati and found that they were still 100 per

* I am indebted to Dr. Carl Ten Broeck for supplying the 23 females and 9 males to start my colony of PRI mice.

cent resistant to the 17 D yellow fever virus. Since Swiss mice are 100 per cent susceptible to this virus, it became possible to carry out definitive tests on the mechanism by which this resistance was inherited, and the results of such a study are summarized in TABLE 1.* Inoculation of the F_1 , F_2 , and various backcross progeny with 17 D virus yielded mortality percentages which corresponded exactly to those expected on the basis that resistance was dominant, susceptibility recessive, and that a single pair of genes was responsible. The serum of the resistant PRI mice had no antibodies for the yellow fever virus before inoculation, but antibodies were present one month after inoculation, even in those receiving an amount of virus equivalent to 1 LD₅₀ for Swiss mice. It was found that the 17 D virus multiplied in the brains of the resistant PRI mice, but the peak titers were only 1/10,000 to

TABLE 1
MECHANISM OF INHERITANCE OF RESISTANCE OF 17 D STRAIN OF YELLOW
FEVER VIRUS MICE TO APPROXIMATELY 10,000 LD₅₀ OF VIRUS INJECTED
INTRACEREBRALLY IN FOUR-WEEK-OLD MICE

<i>Mice used</i>	<i>Observed</i>		<i>Theoretical</i>		
	<i>Number of mice inoculated</i>	<i>Mortality (per cent)</i>	<i>Genetic formula of mice used for breeding</i>	<i>Genetic formula of progeny</i>	<i>Expected mortality (per cent)</i>
Swiss (S).....	300	100	aa × aa	aa	100
PRI (R).....	100	0	AA × AA	AA	0
F_1 (S × R)....	51	0	aa × AA	Aa	0
F_2 (F_1 × F_1)..	213	28.2	Aa × Aa	AA + 2Aa + aa	25
Backcross:					
F_1 × R.....	79	0	Aa × AA	Aa + AA	0
F_1 × S.....	90	50	Aa × aa	Aa + aa	50
Susceptible F_2 × S	21	100	aa × aa	aa	100

* The theoretical calculations are based on the following assumptions and designations: Mendelian autosomal inheritance; one pair of genes for the character; gene for resistance, dominant—A; gene for susceptibility, recessive—a; Swiss mice homozygous for susceptibility—aa; and PRI mice homozygous for resistance—AA.

1/100,000 of that achieved in the brains of the susceptible Swiss, F_2 and backcross mice.

In the case of the 17 D virus, only one factor was involved in the resistance exhibited by the mature PRI mice, *i.e.*, the genetically transmitted factor which inhibited viral multiplication. However, other conditions were found under which mice could possess the factor for inhibition of viral multiplication and yet fail to be resistant. Two types of observations led to the concept of special cellular vulnerability as a modifying factor: (1) Suckling PRI mice up to four days of age failed to resist 17 D virus. The mortality was 100 per cent at one and two days of age and approximately 50 per cent at three days of age. However, the level of viral multiplication in the suckling mice which succumbed was not higher than that achieved in the

* The data summarized in TABLES 1-6 will be published in greater detail elsewhere.

mature, resistant mice. (2) The French neurotropic strain of yellow fever virus* did not behave like the 17 D strain in PRI mice; some died and some lived, a larger number among those inoculated with the smaller doses of virus frequently dying (TABLE 2). However, the level of viral multiplication in the mice which succumbed was not higher than the peak titers achieved in those which resisted either the French neurotropic or the 17 D strains (TABLE 3).

TABLE 2
BEHAVIOR OF TWO DIFFERENT STRAINS OF YELLOW FEVER VIRUS UPON
INTRACEREBRAL INJECTION INTO THE GENETICALLY-DISTINCT
PRI AND SWISS MICE

Strain of virus	Number of mouse passages in Swiss mice	Mice inoculated	Mortality at indicated dilutions of virus									LD ₅₀ reciprocal of log	Highest level of viral multiplication in brain
			10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹		
17 D Chick embryo vaccine	4	PRI Swiss	0/10 10/10	0/10 10/10	0/10 10/10	0/10 10/10	0/10 10/10	0/10 5/10	— 1/10	— —	— —	— 6.0	1.8 to 3.3* 7.5
French neurotropic	252	PRI Swiss	1/10 10/10	2/10 5/5	3/9 5/5	6/10 5/5	5/9 5/5	4/10 5/5	7/10 5/5	2/10 1/5	— 0/5	— 7.6	3.2 to 4.4 8.2

*Reciprocal of log of LD₅₀.

TABLE 3
SPECIAL CELLULAR VULNERABILITY AT LOW LEVELS OF VIRAL MULTIPLICATION
AS A FACTOR IN SUSCEPTIBILITY
INFLUENCE OF AGE OF HOST AND STRAIN OF VIRUS

Strain of Yellow Fever virus injected intracere- brally	Mice		CNS signs and death	Level of virus multiplication peak titers recip- rocal log LD ₅₀
	Strain	Age		
17 D	Swiss	3 weeks	+	7.5
	PRI	3 weeks	0	1.8 to 3.3
	PRI	<4 days	+	2.5 to 3.7
French neurotropic	Swiss	3 weeks	+	7.6 to 8.2
	PRI	3 weeks	76% 0	4.2 to 4.4
	PRI	3 weeks	24% +	3.2 to 3.8

Tests performed on the progeny resulting from crossing PRI and Swiss mice suggested (a) that the special vulnerability of some PRI mice to the French neurotropic virus is also genetically determined, and (b) that the factors responsible for low viral multiplication and high cellular vulnerability are inherited independently (TABLE 4). The fact that 24 per cent of PRI mice die after inoculation of the French neurotropic virus suggests that the majority of PRI mice carry genes which make for lower vulnerability. Since the F₁ (PRI X Swiss) progeny exhibited a highly significant

* I am indebted to Dr. Max Theiler of the Laboratories of the International Health Division of The Rockefeller Foundation for supplying me with both strains of the yellow fever virus.

increase in mortality, it is evident that the Swiss mice are especially endowed with the factor or factors which make for greater vulnerability. To obtain additional information on the genetic aspects of the factor of cellular vulnerability, PRI males of proved resistance to the French neurotropic virus were mated either with Swiss females or with PRI females, proved by subsequent tests to be either susceptible or resistant. Since a certain proportion of the progeny of resistant parents proved to be susceptible, it may be postulated that some of the resistant mice were carrying recessive genes for high vulnerability and that low vulnerability was inherited as a dominant. The incidence of susceptibles was approximately twice as high in the progeny of resistant fathers and susceptible mothers as in the progeny of resistant parents. These studies are still incomplete, however, because it has not yet been shown that when both the paternal and maternal PRI mice are susceptible to the French neurotropic virus, all the progeny are also susceptible. From the experiments performed thus far, it appears that, for

TABLE 4
ROLE OF GENETIC FACTORS FOR VIRUS MULTIPLICATION AND CELL
VULNERABILITY IN RESISTANCE OF HOST TO TWO DIFFERENT
STRAINS OF SAME VIRUS

(VIRUS INJECTED INTRACEREBRALLY IN ADULT MICE)

<i>Mice used</i>	<i>17 D yellow fever</i>		<i>French neurotropic yellow fever</i>	
	<i>Number of mice inoculated</i>	<i>Mortality (per cent)</i>	<i>Number of mice inoculated</i>	<i>Mortality (per cent)</i>
Swiss (S).....	300	100	150	100
PRI (R).....	100	0	121	24}
F ₁ (S × R).....	51	0	84	70)*

* $\chi^2 = 44$; $P = <.000,001$.

the French neurotropic virus, the PRI mice are uniformly homozygous with respect to the dominant genes responsible for low levels of viral multiplication and predominantly heterozygous with respect to cell vulnerability. The Swiss mice, on the other hand, are not only homozygous with respect to the recessive genes responsible for high levels of viral multiplication, but also appear to be homozygous for the recessive genes associated with high cell vulnerability.

The genetic factor in PRI mice which inhibits the multiplication of yellow fever virus has been found to have a similar effect on the viruses of dengue fever, West Nile fever, Japanese B and St. Louis encephalitis, but was without effect on a large group of other viruses listed in TABLE 5. Only the dengue viruses (three different strains) behaved like the 17 D strain of yellow fever virus in that all the PRI mice were uniformly resistant regardless of dosage. The behavior of the West Nile, Japanese B and St. Louis viruses varied markedly with the strain of virus and the number of passages it had had in Swiss mice prior to test in PRI mice (TABLE 6). The data shown in this table indicate also that viruses whose multiplication is in-

hibited by the inherited host factor may overcome the host's resistance in one of two ways, either by possessing or developing the capacity to produce lethal effects at low levels of viral multiplication, or by the development of variants which are no longer inhibited by the inherited multiplication-regulating factor. Thus, the early-mouse-passage Korea and Okinawa strains of Japanese B encephalitis kill only a small proportion of the PRI mice, and the level of multiplication in the brains of the mice which succumb is, in general, only about 1/100,000 of that achieved in the brains of the susceptible Swiss mice. The Nakayama strain of Japanese B encephalitis provides an example of a virus which, between the 43rd and approximately 70th mouse passages, acquired the capacity to multiply at a 20,000-times-higher level and, coincidentally, the capacity to kill 100 per cent of the PRI mice. Although the level of multiplication of this 70th mouse passage virus

TABLE 5
SELECTIVE ACTION OF MULTIPLICATION INHIBITION FACTOR OF PRI MICE
ON VARIOUS VIRUSES PROLIFERATING IN MOUSE BRAIN

<i>Action</i>	<i>Virus</i>
Multiplication inhibited PRI mice completely or partly resistant	Yellow Fever Dengue Fever West Nile Fever Japanese B Encephalitis St. Louis Encephalitis Russian Spring-Summer Encephalitis
Multiplication not affected PRI mice fully susceptible	Western Equine Encephalitis Eastern Equine Encephalitis Venezuelan Equine Encephalitis Poliomyelitis Mouse Encephalomyelitis—"TO" Rabies Lymphocytic Choriomeningitis Herpes Simplex Vesicular Stomatitis Rift Valley Fever

in the brains of PRI mice is still not as high as in Swiss mice, and the rate of multiplication, as measured by the incubation period, is still slower in the PRI than in Swiss mice, the PRI mice are no longer protected by their special genetic constitution against this new variant of the Japanese B virus. The "Webster No. 3" strain of St. Louis encephalitis, which has had a large, though unknown, number of mouse brain passages, killed almost all PRI mice at a level of multiplication that is approximately 1/100,000 of that achieved in the brains of Swiss mice. The West Nile "Egypt 21" strain* presents a remarkable example of a change in "virulence" after only one mouse passage. The "Egypt 101" strain, after only two mouse passages, killed 100 per cent of PRI mice, although the level of viral multiplication was only 1/4,000 of that in the brains of Swiss mice. The "Uganda" strain exemplifies a change in virulence between the 25th and approximately 50th

* I am indebted to Doctors J. R. Paul and J. L. Melnick for the Egyptian strains of the West Nile virus.

mouse brain passages and, in this case, the 100 per cent mortality of PRI mice is associated with a high level of viral multiplication.

The analysis of the genetic aspects of host resistance indicated that, although other factors relative to the host and the virus greatly influence the outcome of certain viral infections, the level of viral multiplication is of very great importance. I should now like to present some data which

TABLE 6
BEHAVIOR OF DIFFERENT STRAINS OF VARIOUS VIRUSES WHICH ARE
AFFECTED BY THE MULTIPLICATION INHIBITING FACTOR IN
PRI MICE

Virus	Strain	Number of passages in Swiss mice	Behavior in PRI Mice		Level of multiplication in brains of Swiss mice
			Mortality (per cent)	Level of multiplication on first day of CNS signs	
Japanese B Encephalitis	Korea	4	13	2.4, 3.0, 3.8*	8.0*
	Okinawa	7	26	2.8, 3.6, 5.5	9.2
	Nakayama	43	50	3.3	7.8
	Nakayama	70?	100	7.6	9.5
St. Louis Encephalitis	Winkler	8	6	3.6	7.8
	Webster 3	Very many over 17 years	90	4.2	9.0
West Nile fever	Egypt 21	1	0	—	7.5
	Egypt 21	2	37	—	8.6
	Egypt 101	2	100	5.8	9.4
	Egypt 19	4	25	—	8.6
	Uganda	25	60	—	8.0
	Uganda	50?	100	6.8	8.3
Dengue Fever	Hawaii	114	0	—	8.0
	New Guinea "C"	18	0	—	7.6
	New Guinea "D"	18	0	—	6.8

* Reciprocal of log of LD₅₀.

indicate that the changes in resistance produced by hormones and age may also be associated with changes in the level of viral multiplication.

Although many studies have been carried out on the effect of a variety of hormones on viral infections, none have yielded such striking results as the recent ones with cortisone. The effects on influenza and mumps in eggs⁷ and on one type of Cocksackie virus in mice⁸ have been reported by Kilbourne and Horsfall. Shwartzman's report⁹ of the enhancing effect of cortisone on experimental poliomyelitis in hamsters was confirmed in my laboratory.¹⁰ Using the Lansing strain of virus, we found that the 50 per cent paralytic

titer (PD_{50}) was 10 times greater, and the LD_{50} was approximately 100 times greater in cortisone-treated young hamsters than in the untreated controls. The level of viral multiplication, determined in individual hamsters at the onset of paralysis, was found to be 10 to 100 times higher in the cortisone-treated animals, which usually develop prostrating paralysis and die, than in the untreated hamsters, which usually develop a limited and often transitory type of paralysis.

It should be noted that we have here an example of cortisone exerting its effect on a partially resistant host, the hamster, infected by a virus, the Lansing strain of poliomyelitis, which, in another host, the mature mouse, can produce a severe, prostrating, fatal infection without the benefits of experimentally-added cortisone. When cortisone is supplied to mice, the effect on infection with the Lansing virus is minimal, and may manifest itself only in shortened incubation periods and slightly higher mortality rates among those inoculated with small doses of virus. Striking effects of cortisone can be demonstrated in still another way, however, when an ordinarily susceptible host is infected with strains of virus of low "virulence." Thus, in rhesus monkeys, the effect of cortisone was negligible when highly virulent strains of poliomyelitis virus were used and striking when strains of low virulence were used. Studies recently completed on sufficiently large numbers of monkeys to render statistically significant results indicated that when strains of poliomyelitis virus of low virulence were used, cortisone converted the nonparalytic and mild paralytic infections to severe prostrating paralysis.¹¹

The various ways in which age modifies the response of experimental animals to infection, especially infection with neurotropic viruses, have been described in many reported studies.¹² The recent discovery of the Coxsackie viruses has revealed a whole group of agents whose pathogenicity for experimental animals is markedly influenced by age. Although almost all neurotropic viruses find a better medium for multiplication in the embryonic and early postnatal tissues of susceptible hosts, it is not a general rule. The Lansing strain of poliomyelitis virus finds it more difficult to get started in the brain of newborn mice than in that of mature mice, the amount of virus required to initiate infection in the former being 10 to 100 times greater than that required in the latter.¹³ The recent isolation of a variant strain of poliomyelitis virus¹⁴ which is not affected by this "barrier" to viral multiplication in the brain of newborn mice is a further example of the dynamics of the relationship between viruses and the tissues they parasitize.

The "maturation resistance" to infection with certain neurotropic viruses exhibited by various hosts falls into two categories: a change affecting the central nervous system (CNS) as a whole, in which resistance is demonstrable by intracerebral inoculation of virus; and a change limited to certain tissues or pathways, over which some neurotropic viruses must pass either for initial invasion of the CNS or for subsequent progression within it, a change which can be demonstrated only by inoculation of virus by some peripheral route. The "maturation-resistance," which depends on changes in the pathways pursued by certain viruses, may be highly localized and may

appear at different ages, varying with the animal, pathway and virus. The level of viral multiplication also has an important role here, since the barrier to viral progression has been located in several instances at sites in which the virus multiplied at a lower level in the older, resistant animals than in the younger, susceptible animals.

An example of selective "maturation resistance" affecting the entire CNS is shown in TABLE 7. The viruses of St. Louis and Japanese B encephalitis, injected into the brain of albino rats, kill 100 per cent of the animals when they are only seven to eight days old, about 50 per cent of the animals when they are 12 days old, and none when they are 21 days old or older at the time of inoculation. The virus of vesicular stomatitis, rabies, Western equine and Eastern equine encephalitis, and certain other viruses, on the other hand, are not affected by this change, which occurs in the rat brain at about the 12th day of life, since rats of all ages die after intracerebral

TABLE 7
EXAMPLE OF SELECTIVE "MATURATION RESISTANCE" AFFECTING ENTIRE
CNS—EFFECT OF INTRACEREBRAL INJECTION OF THREE DIFFERENT
VIRUSES IN RATS OF INCREASING AGE

Age of rats (days)	<i>St. Louis Encephalitis</i> *		<i>Japanese B Encephalitis</i> †		<i>Vesicular Stomatitis (N.J.)*</i>	
	Number tested	Mortality (per cent)	Number tested	Mortality (per cent)	Number tested	Mortality (per cent)
7-8	8	100	16	100	6	100
12	15	47	25	64	3	100
21-22	12	0	12	0	18	100
27-90	11	0	15	0	5	100

* From data of DUFFY & SABIN, 1942, J. Bact., **43**: 88; and unpublished data by these authors.

† From data of DUFFY, 1951, Proc. Soc. Exp. Biol. Med., **76**: 566.

inoculation. It has been demonstrated that the resistance of rats to St. Louis encephalitis, which appears at approximately the 12th day of life, is associated with a change in the level of viral multiplication. In the resistant 21-day-old rats, the level of viral multiplication was only 1/1,000 of that achieved in the susceptible seven- to eight-day-old animals.¹⁵ It is of interest to note in this connection that Potter and his associates¹⁶ found that the concentration of certain enzymes in the brain of rats was low during the first six days of life and increased rapidly thereafter. These enzymes were succinic dehydrogenase, cytochrome oxidase and adenosine triphosphatase. It may not be altogether fruitless to speculate on the role which the mere concentration of these or related enzymes may play in inhibiting multiplication of viruses whose pathogenicity for rats is altered as these enzymes increase in concentration. It is also notable that viruses which are affected by this "maturation change" in the brains of rats are also viruses whose multiplication is depressed by the genetic factor in PRI mice while those viruses which are equally pathogenic for mature rats are not affected by the genetic factor in PRI mice. Is it possible that the special

gene which inhibits the multiplication of these viruses in the brains of PRI mice is also responsible for a much higher concentration of the above-mentioned enzymes? A study of the relative concentration of these enzymes in the brains of PRI and Swiss mice would be of special interest, therefore. Do we have here a clue to a group of enzymes that is important for the multiplication of one group of viruses and not for another? The factor or factors which can limit the multiplication of this group of viruses may, of course, have nothing to do with these particular enzymes, but it appears to be a clue that is worth investigation.

Summary

Natural resistance depends on changes in the host which affect different viruses in different ways. The genetic constitution of the host is important in natural resistance by determining the level of viral multiplication as well as the vulnerability of the tissues. The mechanism of inherited resistance to at least one mammalian virus (yellow fever, 17 D) is Mendelian in character and depends on a single pair of genes which depress the level of viral multiplication. Evidence indicates that special cellular vulnerability of the host, influenced either by age or genetic constitution, can eliminate the resistance gained from inheritance of the factor which holds viral multiplication at a low level. Variants of the same virus may abolish the inherited resistance of the host either by possessing or developing the capacity to kill at lower levels of multiplication, or by overcoming the barrier to higher levels of multiplication imposed by the inherited factor. Hormones, *e.g.*, cortisone, and changes produced in the tissues by age also alter the response of the host to certain viral infections. Here, too, resistance occasionally has been correlated with low levels of viral multiplication, and susceptibility with high levels.

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VIRUSES WITH ONCOLYTIC PROPERTIES AND THEIR ADAPTATION TO TUMORS

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Viruses are called oncolytic when they destroy tumors. This ability is not surprising since viruses are well known for their destructive effect on specific normal tissues. Oncolysis, by viral action, has not been studied very thoroughly until recently because earlier workers put more emphasis on the growth of the virus in the tumor rather than the effect of virus on tumor growth. Those of us who have been interested in the oncolytic capacity of viruses now have some knowledge, admittedly very incomplete, of the circumstances under which this takes place.

Oncolysis can be demonstrated by examining what happens when mice bearing the transplantable mouse sarcoma 180 are inoculated intraperitoneally with approximately 10,000 LD₅₀ of Russian encephalitis virus.¹ The tumors grow for a short time but, after three days, retransplantation of tumor pieces into virus immune animals† shows only 50 per cent tumor growth in contrast to 100 per cent growth in the controls. Six days later, all transplanted tumor pieces fail to grow and histological examination shows only necrotic, non-viable tissue (FIGURES 1 and 2). The originally-inoculated tumor-bearing animals become paralyzed and die after seven or eight days. The oncolytic ability of the virus, however is not dependent on the susceptibility of the host to infection, as can be shown by the following examples of tumor destruction with host survival: (1) Sharpless, Davies and Cox² have shown that the chicken tumor RPL 12 is destroyed in much the same way as the sarcoma 180 by the viruses of Russian encephalitis, louping ill and St. Louis encephalitis; (2) Ginder and Friedewald³ have demonstrated the disappearance of the rabbit fibroma by the intramuscular inoculation of Semliki Forest virus; and (3) recently, in our laboratory, Russian encephalitis virus has destroyed the sarcoma 180 grown in irradiated rats.

The conditions under which the sarcoma 180 is destroyed by the virus of Russian encephalitis, the tumor-virus system which we have studied most thoroughly, are described here. The adverse tumor effect is dependent on the presence of active virus and therefore does not take place in either actively or passively immune animals. Virus inactivated by either formalin, heat or ultraviolet rays has shown no effect on tumor growth so far. The general rule may be established that the tumor is protected when the animal is protected against the virus and the tumor is destroyed when the animal is unprotected. Active virus is the first requisite, therefore.

The second requisite is time. A certain number of days is required for

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† It is necessary to transplant tumor into virus-immune animals because the tumor pieces carry so much virus that other animals would die of the infection. The immune state of the animals has no effect on the tumor growth.

the destruction of a tumor. FIGURE 3 shows the distribution of virus in the blood, brain and tumor of mice killed daily after the intraperitoneal inoculation of 10,000 LD₅₀ of Russian encephalitis virus. Although the virus appears in high titer in the tumor tissue as much as one day after inoculation, increases until it reaches a maximum three days after inoculation and then remains at this high level, complete tumor destruction does not take place until the sixth day. The amount of time necessary for the effect is dependent on the amount of virus inoculated. If huge quantities (1,000,000 LD₅₀'s, equivalent to the 10⁻¹ dilution shown in FIGURE 4) are inoculated intraperitoneally, complete tumor destruction is accomplished in three days. When small quantities (100 LD₅₀'s, equivalent to the 10⁻⁵ dilution shown in

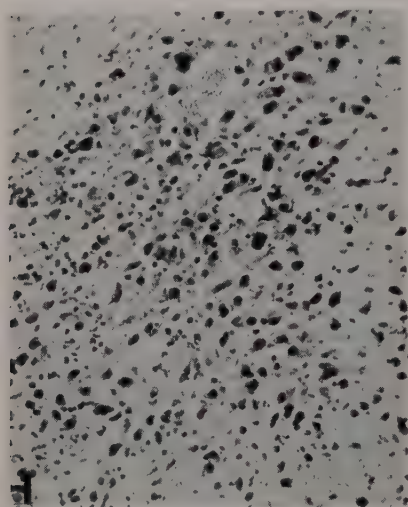


FIGURE 1. Histological appearance of sarcoma 180 removed from a mouse nine days after infection with Russian encephalitis virus (H. & E. stain).

FIGURE 2. Same age sarcoma 180 removed from a non-infected mouse.

FIGURE 4) are inoculated intraperitoneally, complete tumor destruction is accomplished in eight days. The age or size of the tumor apparently make no difference in its susceptibility to destruction. Also, the virus is an effective agent whether it is inoculated intracerebrally, subcutaneously, intraperitoneally or directly into the tumor.⁴

In a survey of other viruses and other tumors for their oncolytic abilities one is immediately faced with another problem. Each virus appears to differ considerably and specifically in its ability to destroy different tumors. For example,* if three viruses are tested for destructive effects on different mouse tumors, it is found that each virus has a different tumor spectrum (TABLE 1). Of the six tumors tested, Russian encephalitis and West Nile viruses destroy four out of six, but the latter virus is relatively ineffective

* The data for the West Nile and Bunyamwera viruses were obtained from Koprowski and Norton.⁵

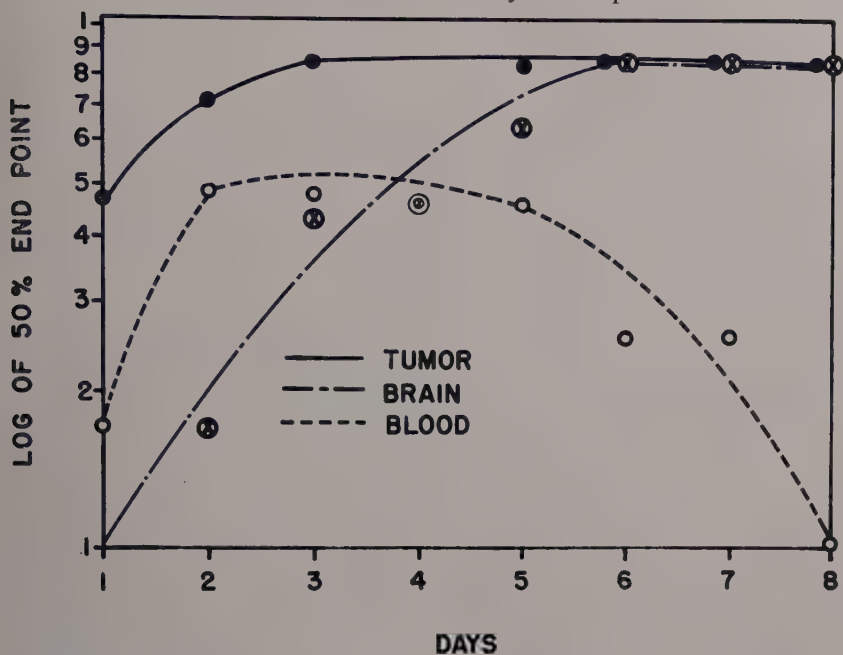


FIGURE 3. Amount of virus in blood, brain, and tumor following intraperitoneal inoculation of Russian encephalitis virus into mice bearing sarcoma 180.

DESTRUCTION OF THE SARCOMA 180 IN RELATION
TO THE AMOUNT OF RUSSIAN ENCEPHALITIS
VIRUS INJECTED INTRAPERITONEALLY.

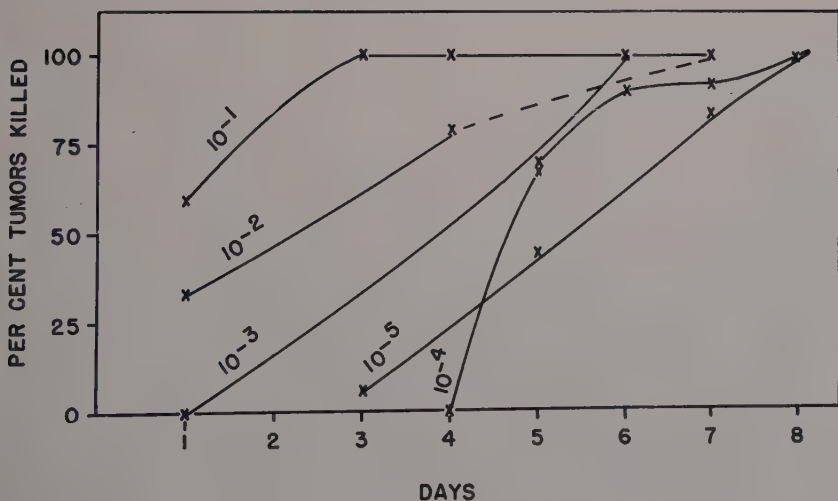


FIGURE 4

against EO 771, while affecting the Wagner tumor, which is not inhibited by Russian encephalitis. The Bunyamwera virus is effective against the two osteogenic sarcomas, relatively ineffective against EO 771, and affects no others. Oncolysis, therefore, is a very specific affair and it is difficult to say that a virus has no oncolytic properties, for fear that it has not been tested against the proper tumor.

A review of the old and recent literature shows that a great number of viruses have been tested against various tumors in many different species. It is impossible to do more here than list those which have some oncolytic

TABLE 1
TUMOR DESTRUCTION BY VIRUSES

Virus.....	Tumor type					
	<i>Sarcoma</i>	<i>Carcinoma</i>	<i>Osteo. sarcoma</i>	<i>Osteo. sarcoma</i>	<i>Sarcoma</i>	<i>Melanoma</i>
	S-180	EO 771	Ridgway	Wagner	MCI	Harding-Passey
R.E.....	+	+	+	—	+	—
W.N.....	+	±	+	+	+	—
Buny.....	—	±	+	+	—	—

TABLE 2
VIRUSES FOUND TO BE EFFECTIVE AGAINST ONE OR MORE ANIMAL TUMORS

<i>Virus</i>	<i>Tumors tested</i>	<i>No. positive</i>	<i>No. negative</i>
RSSE.....	14	9	5
L.I.....	9	5	4
W.N.....	8	5	3
Ilheus.....	8	3	5
St. Louis.....	6	3	3
Bunyamwera.....	8	3	5
V.E.E.*.....	6	3	3
S.F.V.....	5	1	4
Av. Pest.....	4	1	3
Vaccinia.....	13	3	10
Virus III.....	3	1	2

* Erratic virus.

properties, and by listing the numbers of tumors with which they have been tested, give some indication of their effectiveness (TABLE 2).^{*} Such a list, of course, gives only a rough approximation of the degree of the oncolytic properties of the viruses, since not all the experiments have been done under the same conditions or with the same tumors. Altogether, 11 viruses have been effective against one or more tumors. Those with the highest proportion of effectiveness have been neurotropic. In fact, it may be that the vaccinia and virus III viruses act by a different mechanism, since their ac-

^{*} These data have been compiled from the literature and also from the unpublished results of Koprowski, Sharpless, and Southam, and from our laboratories.

tion is slow and incomplete. TABLE 3* lists viruses which have been tested against one or more tumors and found ineffective. The failure of these agents to affect tumor growth is not due to their inability to multiply in tumor tissue since many attain titers comparable to the oncolytic viruses. For some reason, however, their presence has no effect on tumor growth, and some have been transferred along with the tumor for many generations, as in the virus III-Brown Pearce combination.⁶

We have been interested in determining whether it is possible to change a virus as far as its oncolytic or neurotropic properties are concerned. The virus chosen for this work was Russian encephalitis, since the conditions under which the parent or "stock" strain destroys tumors had been defined. Serial passages were begun in three different tissues: (1) brain to brain; (2) sarcoma 180 to sarcoma 180; and (3) Wagnar osteogenic sarcoma to the same tumor. The last tumor resisted destruction by this virus in many previous experiments. Passages were made at bi-weekly intervals and every

TABLE 3
VIRUSES FOUND TO BE INEFFECTIVE AGAINST ONE OR MORE ANIMAL TUMORS

<i>Neuro group</i>	<i>Neuro group</i>	<i>Miscellaneous</i>	<i>Rickettsia</i>
Rabies.....	Coxsackie (A)	PR8	R.M. Spotted
Y.F.....	Lansing polio	Neurotropic flu	N. Queensland
Herpes.....	Anopheles B.	Swine flu	Tick typhus
Jap. B.....	Dengue	Mumps	Rickettsial pox
E.E.E.....	Kumba	NDV	Boutonneuse fever
W.E.E.....	Bovine enceph.	L.G.V.	S. Afr. tick
MM & ColSK.....	Leucocelaneus	Feline pneu.	Epidemic typhus
Bwamba.....	Sabethes	Canine dist.	Murine typhus
GD VII.....	Passos I	Canine inf. hep.	
Uganda S.....	Passos II	Hog cholera	
Zikka.....			

tenth passage was tested for any change in its oncolytic ability (FIGURE 5). The "stock" or parent strain had no effect on the growth of the transplanted pieces of sarcoma 180 one day after inoculation, and was capable of destroying 50 per cent of the sarcoma in three days. After 20 to 30 passages in the sarcoma 180, the virus was capable of destroying all of it in three days. Further passage resulted in an increase in its ability to attack neoplastic tissue until we now have some strains which will destroy all of the tumor 24 hours after intraperitoneal inoculation. In contrast, the same parent virus, after continuous passage in brain tissue, has gradually lost most of its oncolytic ability so that after 117 passages only 30 per cent of the sarcoma 180 was destroyed seven days after inoculation, although the parent virus completely destroys the tumor in this length of time. It can be demonstrated, therefore, that the oncolytic property of this virus in regard to the sarcoma 180 can be changed in either direction. Its neurotropism has not changed significantly. A few mice, in the sarcoma 180 to sarcoma 180 passages, have survived, even though they received tremendous amounts of virus (100,000,000 LD₅₀). They have been immune both to reinoculation

* See footnote, p. 948.

of virus and tumor. This has happened seldom, however, and is difficult to repeat.

When the Wagner virus was tested at various intervals, a change in its behavior toward the tumor appeared after 40 passages (TABLE 4). On further passage, its oncolytic power increased until it was capable of destroying all the tumor, although the parent virus is seen to be ineffective.

Per cent of Sarcoma 180 destroyed at one and three days following intraperitoneal inoculation of 0.05 c.c. of 10^{-3} dilution of Tumor to Tumor and Brain to Brain Passage.

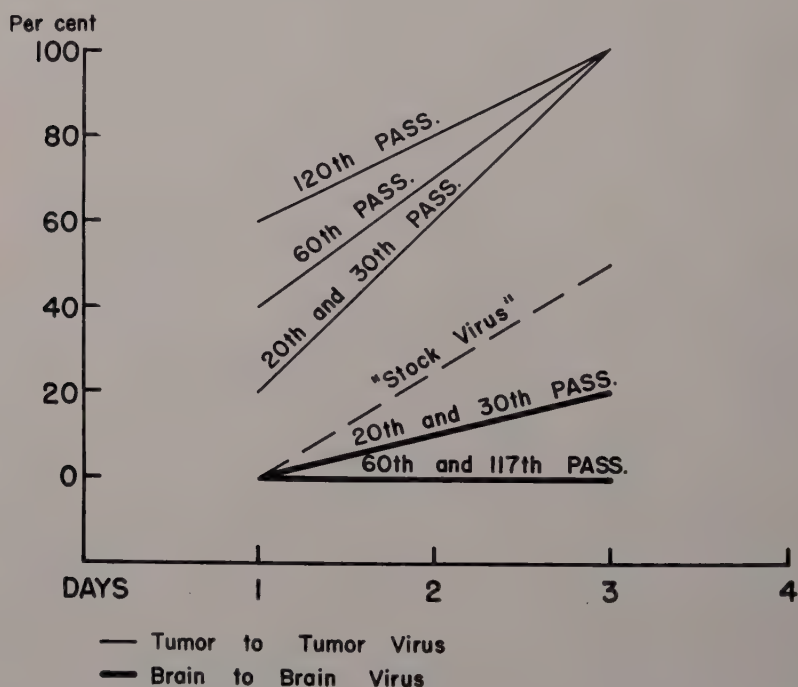


FIGURE 5

These experiments illustrate that a virus can be changed by continuous passage in tissue so that its ability to destroy a susceptible tumor is increased or decreased, or a strain may be developed which destroys a hitherto non-susceptible tumor.⁷

Both parties involved in the oncolytic process can be examined in an effort to ascertain the mechanism of action. It has been shown that different tumors react differently to the same virus. This is unrelated to the histological type of the tumor, the same virus readily destroying one carcinoma and not another, or one osteogenic sarcoma and not another. It is ques-

tionable whether the growth rate of the tumor is related to its resistance to destruction, although this is a possibility. For example, tumors which require six weeks to two months to appear in the mouse have been unaffected by the Russian encephalitis virus, although it has multiplied readily in all but one of them. On the other hand, at least two rapidly-growing tumors are unaffected by this same virus. It is doubtful if the growth rates are the only reason for the differences noted.

The viruses may be classified as follows: (1) those which grow in a tumor and do not affect it; (2) those which grow in a tumor and destroy it; and (3) those which fail to grow in tumors. There are two rather striking examples of this latter situation. The virus of Russian encephalitis fails to multiply in the melanoma although it does so readily in every other type of tumor tissue we have studied. Koprowski and Norton⁵ have also reported the failure of the Eastern equine encephalitis virus to multiply in the sar-

TABLE 4
COMPARISON OF WAGNAR PASSAGE VIRUS AND "STOCK VIRUS" ON THE
GROWTH OF THE WAGNAR TUMOR

Passage number	Titration i.c.	Titer		Per cent of tumor destroyed
		Tumor	Brain	
"stock virus".....	9.75	6.5	8.0	0
41st passage.....	7.5	6.0	6.0	20
"stock virus".....	8.5	8.0	7.5	9
55th passage.....	9.0	8.0	8.5	64
"stock virus".....	8.5	6.5	8.0	0
79th passage.....	9.5	8.5	8.5	100
"stock virus".....	10.0	6.5	8.0	0
90th passage.....	10.0	7.5	8.5	100

coma 180 although it grows readily in the Ridgway osteogenic sarcoma. It should be noted that at least two sets of viruses which are closely related immunologically have the same tumor spectrum, *i.e.*, the Russian encephalitis and louping ill viruses, and the West Nile and Egypt viruses. One other set of viruses known to have common antigenic components, the St. Louis and Jap B viruses, have not always shown the same affinity for tumor.

It seems, therefore, that the all-important factor in oncolysis is the virus-tumor combination itself. The mechanism of tumor destruction by virus is unknown. Since most, if not all, of the effective viruses are neurotropic, it seems possible that there are some common metabolic product or products in both tumor and brain which these viruses find necessary for their support and, as a result of the competition for it, the host cell is destroyed. In the adaptation experiments, we can only speculate on the mechanism. The variant strains may be produced by simple selection or viruses may change their chemical make-up by the process of passage in one type of tissue so that they may better compete with the host cell. All definite answers to these questions must await a more thorough understanding of virus-host relationships.

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IMMUNOLOGICAL BARRIERS TO VIRAL GROWTH

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The characterization of certain antibodies as "virus-neutralizing" indicates that they have a direct and measurable inhibitory effect on infection with the virus that has stimulated their production. This is a strictly functional definition which tells nothing of the nature of the specific viral constituents with which an antibody reacts. Because of their complex structure, it is probable that all viruses contain more than one prosthetic group which can act as distinct antigens. There is no reason, therefore, to believe that neutralization of a virus suspension should necessarily be due to a single homogeneous type of antibody. More likely, effective neutralization may be supposed to depend on the blocking of a sufficient number of antigenic groupings and a variety of reactive spots, each of which in turn may have a specific role to play in the process of infection and multiplication. We know from work by Smadel, Hoagland and their co-workers (reviewed by Smadel and Hoagland, 1942), and by others, that purified elementary bodies of vaccinia virus yield at least three specific and chemically-defined antigenic constituents (the L and S protein and the nucleoprotein fractions), none of which has stimulated production of virus-neutralizing antibody. Does this mean that none of the three fractions is essential to the successful completion of the infectious process, or that the complexes which they form with their antibodies are easily dissociable in the susceptible host, or that the immune sera which have been tested are not sufficiently potent to saturate all of the reactive groups on the virus particle?

These open questions are indicative of our ignorance concerning the specific nature of those viral constituents which play a vital role in the initiation and consummation of infection of cells. We may hope, eventually, to gain better insight into this problem by continued efforts to analyze the kinetics and mechanism of viral growth, and by studying the effect of antibodies on specific phases of the growth process. Beginnings in this direction have been made in recent observations on bacteriophages and on influenza viruses. These two types of viruses have lent themselves to reliable quantitative investigation because sensitive biological assay methods are available, and because the viruses can be studied in association with isolated host cells or with a homogeneous cell population, such as the entodermal layer of the chorio-allantoic membrane of embryonated eggs. In both systems, physical separation of cells from their liquid menstuum can be achieved at any desired stage in the infectious cycle. Hence, one can determine the rate at which virus particles are removed by the cells from the environment and also measure the release of newly-produced virus into the environment. By adding immune serum or other biologically active substances to the liquid medium, or omitting them from it, one can discern their effect on specific phases of viral multiplication, especially their ability

to disrupt the virus-cell complex. A reasonably consistent concept of the viral growth process has emerged from work on phages and influenza viruses: (1) The union of virus and susceptible cell is followed instantaneously by "disappearance" or loss of identity of the infecting virus particles. (2) The reproductive phase of infection consists of stagewise assembly of new viral particles, infectiousness apparently being the attribute with which newly formed particles are endowed last. (3) Newly produced particles appear after a characteristic lag period and then may be released from infected cells either by lysis or by some other mechanism.

In what way can antibody affect this chain of events? It has been reported that a single, free (extracellular) particle of T2 phage can combine with as many as 5,000 antibody molecules, although an average of 90 molecules per particle is enough to render a virus suspension incapable of multiplication (Hershey, Kalmanson and Bronfenbrenner, 1934). Whether or not coating of a virus particle with antibody interferes with its adsorption on the bacterial host depends probably on the number of antibody molecules and on the particular antigenic prosthetic groups to which they are attached. Hershey and Bronfenbrenner (1948) found that adsorption of T2 on *E. coli* is undiminished even when infectivity is reduced by 90 per cent through the action of antibody. Inactivation of such an order probably means that individual viral particles have combined with only relatively few antibody molecules and that the viral groupings responsible for adsorption on the host cell were not completely covered. This is in substantial agreement with earlier reports by Burnet *et al.* (1937) who found that partially neutralized viral particles were not only adsorbed but also retained the ability to reproduce. If neutralized virus remains adsorbable on host cells under certain conditions of concentration, one would have to postulate that antibody may act on these particles by blocking or by combining with groupings which are essential at a step of the infectious cycle subsequent to adsorption. Delbrück has supplied indirect evidence indicating that antibody can react with such viral constituents. He found (1945a) that under ordinary conditions, *i.e.*, in infection of *E. coli* with a single strain of phage, antibody was entirely without effect on the viral growth cycle when as little as 90 seconds were allowed for adsorption of virus on host cells. One-step growth curves obtained in the presence or in absence of antibody were identical in all characteristics.

Nevertheless, it was possible to show, under the following special circumstances, that antibody could react with phage even after the interaction between virus and host cell had gone beyond the adsorption phase. Delbrück (1945b) found that when reproduction of one virus, *e.g.*, T7, was interfered with by simultaneous infection of the host with another, *e.g.*, T1, the yield of the latter, *i.e.*, the "successful" virus, was depressed by the excluded strain. This "depressor effect" could be mitigated by anti-T7 serum even if the latter was added to the mixed-infected bacteria as late as $3\frac{1}{2}$ minutes after infection. This was remarkable because the "excluded" strain (a term which should be replaced by "suppressed" or "inhibited"), although prevented by the interfering infection from multiplying, was found not only to

be adsorbed, but also to "disappear" after adsorption, just as it would after attachment to normal bacteria. Despite this loss of identity, specific antibody could react with it and thereby inhibit its effect on the yield of the interfering virus.

These findings suggest that during the "normal" growth cycle the infecting virus is removed out of reach of extracellular antibody, or deprived of its antigenic identity almost immediately upon adsorption on the host cell and that this process, whatever its nature, appears to be blocked when reproduction of a phage is inhibited by the mechanism of interference. This concept, based on indirect evidence, would fit in nicely with the findings of French *et al.* (1951) on the inhibition of breakdown of P^{32} -labelled T2r + phage (measured in terms of the amount of isotope converted into a form soluble in 5 per cent trichloroacetic acid) after adsorption on cells previously infected with serologically unrelated T phages.

Work on the effect of antiserum on the adsorption of influenza virus on red cells and on its growth in the chorio-allantoic membrane of embryonated eggs has revealed that at least one animal-pathogenic virus reacts in a manner not unlike bacteriophages. It was first shown by Hirst (1942) that immune serum specifically inhibited hemagglutination by homologous virus and its adsorption on red cells. The demonstration of receptor substances on red cells and the presence of similar substances in susceptible tissues has suggested a basic similarity in the mechanism of adsorption of influenza viruses on red cells and on tissue cells. It is reasonable, therefore, to assume that the neutralizing action of antiserum may be due to combination of antibody with the viral moiety which mediates adsorption on cellular receptors. An alternate mechanism is suggested, however, by the conclusion of Walker and Horsfall (1950) that the neutralizing and the hemagglutination-inhibiting antibodies may be distinct. Regardless of whether or not adsorption is necessarily prevented by neutralizing antibody, it is clear from the work of Henle and Henle (1949) that influenza virus, like phages, escapes the action of antibody once the growth cycle in conjunction with the cell has begun. When immune serum is inoculated into the allantoic cavity 30 minutes after a large dose (10 to 10^9 E.I.D.) of active virus, its immediate effect is to reduce the infectious titer of membrane suspensions by a factor of about 1,000. This is presumably due to its action on superficially adsorbed viral particles, and not on those particles which give rise to progeny, the infecting particles in the strict sense. That the latter are not inactivated is clear from the fact that the yield of newly produced virus at the end of the first growth cycle is the same in serum-treated as in untreated eggs.

It is clear, then, that the action of antibody is limited to its effect on extracellular virus or, perhaps, on superficially adsorbed particles and on particles which, for unknown reasons, do not participate in the generation of progeny. It is inert in relation to virus particles irreversibly committed to the process of reproduction.

The advance in our knowledge on antibody as a barrier to the growth of bacteriophages and influenza viruses, especially in relation to specific phases

of the infectious cycle, is facilitated by the fact that they can be studied in hosts which are themselves incapable of antibody formation. Virus multiplication, therefore, is not "normally" impeded by the action of antibody, and the effects of added immune serum can in turn be accurately quantitated. This is not the case in infections of antibody-producing vertebrate hosts. Here, viral multiplication and the host's immune response may operate simultaneously as competing forces. One can never be quite sure whether virus titrations carried out at different periods give an accurate picture of the extent to which viral multiplication has gone, or whether they measure only those amounts which are demonstrable in excess of neutralizing antibody. Moreover, separation of host cells from their menstrium is difficult to achieve and, therefore, the observations on viral growth and on antibody formation have to be made on homogenized organs or on body fluids. Despite these obstacles, a variety of data recently obtained can be interpreted as compatible in principle with the findings on phages and influenza viruses.

It has long been recognized that the effectiveness of a host's immune response in overcoming infection is determined principally by the quantitative relationship of infecting virus to antibody available. This applies regardless of whether antibody is actively produced or passively administered. A great deal of confusion stems, however, from the practice of depending solely on antibody levels in the circulating bloodstream in gauging a host's response to infection. The antigenic impetus of the infecting dose itself and the importance of antibody supply at the focus of viral multiplication have not been taken into sufficient account. As long ago as 1937, Burnet, Keogh and Lush, in commenting on the perplexities of the immunity problem in experimental poliomyelitis, stated: "There are certain aspects of active immunity to viruses of such limited tissue specificity which bear on our problem of the site of formation of antibody. . . . When a monkey recovers from a paralytic attack of the experimental disease it is completely immune to intracerebral or intranasal re-inoculation with the same strain of virus, and shows virus-inactivating antibody in its serum. On the other hand when animals are given a prolonged series of intradermal injections of virus they develop circulating antibody in considerable amount but are still normally susceptible to infection by virus administered intranasally. From these experiments we have clear evidence that the presence of circulating antibody is in itself not sufficient to protect the animal against intranasal infection. There is very good ground for assuming that all "specific" immunity is dependent on the existence of antibody, either circulating or in the tissue fluids, plus the power to produce further supplies of antibody with increased rapidity on restimulation by the specific antigen. To account for the differences between the two cases we are almost compelled to postulate a local accumulation of antibody, or of the antibody-producing mechanism at some 'strategic points' in relation to the central nervous system. The details of these local mechanisms must be left for future research to elucidate."

Although more recent investigators have succeeded in effectively immunizing monkeys, even by peripheral vaccination, the basic problem referred

to by Burnet *et al.* is as acute now as it was then. Experimental confirmation of their postulate is suggested by the findings of Morgan (1947) in experimental poliomyelitis, although the antibody nature of the virus-inactivating substance extracted from nervous tissue of convalescent monkeys would merit further corroboration. The paramount importance of local antibody response has been demonstrated in studies on another experimental neurotropic virus infection, *i.e.*, equine encephalomyelitis (E.E.) in mice (Schlesinger, 1949a, b). A brief recapitulation of the findings on this model is germane to the present discussion because it has revealed the close interdependence of two competing forces within the central nervous system, *viz.*, (a) rate of viral multiplication and (b) rate of local immune response. Two substrains of the same virus (Western E.E.) differ by their rate of multiplication in mouse brain. One, the R.I. strain, as a result of continued serial passages through mice, has acquired a growth rate twice as rapid as a less well-adapted strain (Kelser). The survival time of mice after intracerebral inoculation of the two strains differs correspondingly. There exists no demonstrable serological difference between the two strains. If mice are vaccinated with large doses of formalin-inactivated virus of either strain, enough to stimulate high serum antibody titers, they survive large or small intracerebral challenge doses of either strain. If the dose of vaccine is reduced, however, so that little or no antibody is demonstrable, they survive challenge with the slowly multiplying strain although they are not protected against comparable amounts of the fast R.I. strain. This difference in response is reflected in the growth rates of the two strains in previously immunized mice. The R.I. strain multiplies at about the same rate as in non-vaccinated control animals. The growth rate of the Kelser strain, on the contrary, is markedly depressed although not entirely inhibited. The virus is demonstrable in the brain at fairly high titer up to the fourth day. On the fifth day, it is replaced by an excess of neutralizing antibody which, for at least four months after challenge inoculation, persists at a high level, far out of proportion to the "physiological" serum antibody/brain antibody ratio (approximately 200/1, as compared with less than 10/1 in challenge survivors). This potent secondary local antibody response is apparently stimulated by the challenge inoculum itself. If graded amounts are inoculated, one finds that mice immunized with small doses of vaccine survive large challenge doses but may succumb to minimal amounts. This "paradoxical" response can best be explained by assuming that in the latter case the amount given is so small that it is immediately utilized in infecting susceptible cells, the local antibody concentration being too low to prevent this. With large challenge doses, on the other hand, enough virus remains free to act as an antigenic booster to stimulate the local antibody-producing elements. The main reasons for believing that this is due to local formation of antibody rather than to non-specific accumulation are (1) its demonstrated specificity in doubly immunized animals and (2) the long persistence of the local antibody excess. The antibody nature of the neutralizing substance is evident from the fact that its titer in relation to the serum titer is closely paralleled by the CNS/serum ratio of complement-fixing antibody. Here, then, is a clear demonstration of the delicate balance between viral

growth rate and local concentration of antibody, where the fate of the animal depends on whether the virus multiplies too rapidly to allow effective antibody production or slowly enough to permit the latter to intervene successfully. Relationships very similar to these are suggested by the work of Karzon and Bang (1951a, b) on the difference in response of chicks to two strains of Newcastle disease virus. Confirmation of the significance of local antibody concentration, if not necessarily local production, comes also from the studies of Fazekas de St. Groth and Donnelley (1950) on immunity of mice to intranasally instilled influenza virus.

In the study of immunity to viruses, more so than in work on antibacterial immunity, the local antigenic stimulus exerted by the challenge or by the infecting dose, as well as the variability of viral growth rate, have usually been overlooked in explaining certain apparent inconsistencies, and immunity has been looked upon as a static rather than a dynamic alteration in the host's behavior. It seems reasonable to suggest that the "masking" of tumor viruses in homogenized tumor tissues and the spontaneous regression of certain virus-induced tumors may be related, in part at least, to the effects of local antibody. This approach has not been fully explored.

Thus far, this discussion has been concerned solely with the effect of neutralizing antibody as a barrier to viral multiplication. Perhaps this is justified because we know of no other immunologically defined entity which displays a similar activity. The question arises, however, of whether the customary concept of immunological specificity as being synonymous with specific antigenic activity may not be too narrow when applied to viruses. Evidence is accumulating to suggest that not only the antibody-producing apparatus but also the host cells themselves are capable of distinguishing between viruses of different antigenic constitution, no matter how closely related they may be in other respects. For plant viruses, this has been recognized for a long time. Although antibody-like substances apparently play no role in resistance of plants to infection, *i.e.*, in suppression of viral growth, acquired immunity is characterized by cross protection limited to strains which are also serologically related. More recently, the study of interference phenomena among bacteriophages, as well as between strains of influenza viruses in host systems incapable of antibody formation, has revealed striking differences in the behavior of antigenically related and unrelated pairs of viruses. Mutual exclusion occurs between serologically distinct phages, while related strains can grow simultaneously in the same cells. In the case of influenza virus, a relation of interference to immunological type specificity is suggested by the difference between the effects of homologous and of heterologous ultraviolet-irradiated strains on a viral growth process already in progress, as described by Henle and co-workers (1947, 1949). It is generally believed that the mechanism of interference may involve competition by two viruses for some cellular constituents essential to viral reproduction. If this is correct, one may postulate that the specific reactive groups which enable certain viruses to utilize one or the other of these hypothetical constituents might be related to those which determine their immunological group relationship, or that immunological relationship in these cases reflects close kinship in the genetic make-up of the viruses.

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INHERITANCE IN BACTERIOPHAGE*

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The unit of genetic experiments is the individual organism. The unit of genetic thinking is the concept of the gene. I propose to trace briefly how one arrives at the concept of the viral gene by genetic experiments with the bacteriophage T2.

The Resting Phage Particle. The individual organism in this instance is the resting phage particle, so-called because it is devoid of all biological activities in the isolated state. The genetic properties of this organism, as of all organisms, are deduced from progenies. T2 belongs to a unique biologic class because of its small size, its stable resting stage, and because its progenies must be obtained from inside the cells of a foreign species, the colon bacillus. These are the facts we have in mind when we use the word virus. They are inconvenient facts, sometimes, but they do not complicate genetic experiments in any essential way. The association between virus and host seems to be one of physiologic convenience. No genetic interaction between them can be detected. We satisfy our conceptual needs by imagining an intracellular, vegetative form of the virus possessing genetic continuity with the resting particles.

Absolute Purity and Absolute Infectivity. It is possible to prepare isotopically marked populations of T2 of which 85 per cent of the phosphorus or 95 per cent of the sulfur (Hershey and Chase, unpublished) are removed from the suspension by specific attachment to sensitive bacteria. Such preparations can be considered chemically pure, and similar preparations are found by microscopic examination to consist entirely of characteristically organized particles. The particles may be counted and their number corresponds within a factor of two or less with the infective titer obtained by counting plaques (Luria, Williams, and Backus, 1951). These facts are prerequisite to genetic experiments, *i.e.*, they show that a plaque comes from a single phage particle, and that genetic inhomogeneities detectable in single-plaque lines of virus must result from accidents occurring among the descendants of a single organism.

Independent Mutations. Starting with a stock of T2 (obtained, for example, by three successive single-plaque isolations on *Escherichia coli* strain B), it is possible to reveal statistically predictable genetic accidents in the following ways:

(1) If 10^9 particles of T2 and about 10^8 cells of *E. coli* strain B/2 (resistant to T2) are incubated together on the surface of an agar plate, a few plaques are likely to appear. Phage particles isolated from these plaques differ from T2 in that their progenies are capable of lysing B/2 (Luria, 1945). The number of such variants found in a T2 stock is characteristic of that stock, not of the stock of B/2 on which it is plated (Luria, 1945). The accident occurs, therefore, in the infected bacterium, and the effects preserved in the

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aberrant progeny are our only source of information about the accident that produced them.

(2) If a few thousand particles of T2 are allowed to form plaques on agar plates seeded with sensitive bacteria, several of the plaques are likely to differ visibly from the majority. The exceptional plaques contain phage particles forming plaques of the aberrant type (Hershey, 1946). These variants, like the ones capable of lysing B/2, result from accidents happening to the virus, not to the prospective host.

Accidents resulting in stepwise heritable changes in the character of the virus are called viral mutations. The two mutants I have just described are called T2h and T2r, respectively. These two mutations occur independently of each other, that is, *r* mutations occur either in T2 or its *h* mutant, and *h* mutations occur either in T2 or its *r* mutant. These facts are conveniently expressed by saying that the *h* and *r* mutations occur at different mutative loci.

All the hereditary variations that have been observed in pure lines of T2 share this same accidental, stepwise character. These mutations cannot be directed by experimental means and so far we have not even learned how to alter the over-all natural frequency of mutation.

Selection and Genetic Equilibrium. The *h* mutant is isolated from T2 by selection on B/2. The *r* mutant is isolated from T2 by manual selection. It is evident that T2 is really a name given to a viral mixture in which a given hereditary type predominates. The same is true of T2h and T2r. If either one is propagated sufficiently long in broth cultures of B, the population tends to regain the composition characteristic of T2, evidently by selection of the respective back-mutants. This is another way of saying that in the course of its laboratory history of propagation on broth cultures of B, T2 has emerged as the best adapted of its alternative hereditary forms to this particular situation.

Genetic equilibria in viral populations are jointly determined by the kinds and frequencies of mutation, and by the working of selective influences. In general, when a viral population is transferred from one host to another, we expect its qualitative composition (determined by mutation) to remain unchanged, whereas its quantitative composition may be altered profoundly. Selective influences are, in other words, subject to experimental control, a fact of very great practical importance in the preparation of viral vaccines. As compared to selective influences, viral mutations are of lesser practical importance and of greater scientific interest because they are not subject to experimental control.

Genetic Recombination. When a growing bacterium is simultaneously infected with one or more particles of *h* mutant and one or more particles of *r* mutant, it lyses after 21 minutes, liberating a viral progeny of about 500 particles. This progeny contains particles giving rise to wild-type clones and particles giving rise to *hr* clones, as well as particles belonging to the two parental types (Hershey and Rotman, 1949). This result defines the combinative locus; we say that the mutations *h* and *r* occur at different combinative loci, and that different combinative loci assort in all possible combinations during the vegetative phase of the virus. Recombination can

be observed only when the viral population contains individuals genetically marked in at least two ways at two or more different combinative loci. The phenomenon just described is called genetic recombination, and the mixed infection with *h* and *r* mutants is called a two-factor cross.

Any two *r* mutants of independent origin will, as a rule, produce mixed infections that have all the characteristics of the two-factor cross $h \times r$ (Hershey and Rotman, 1948). Thus *r*1 and *r*5 parents yield progeny particles giving rise to wild-type and *r*1*r*5 clones. Different *r* mutations tend, therefore, to occur at different combinative loci.

Recombination of an analogous kind can be observed with other mutant characters.

Linkage. Crosses between pairs of different *r* mutants yield progenies containing a proportion of wild-type particles that is characteristic of the pair. These proportions measure the linkage between the corresponding combinative loci in arbitrary (and mathematically complex) units. When the proportion of recombinants is small, two per cent for example, the pair is said to be closely linked. When the proportion of recombinants is large (40 per cent), it can be shown that the loci assort independently, and the pair is called unlinked (Hershey and Rotman, 1948).

The combinative loci, *h* and *r*13, form a closely linked pair. When these two mutants are separately crossed to a number of other mutants, two identical sets of linkage measures are obtained. This is interpreted to mean that the loci *h* and *r*13 lie near each other in the genetic structural material of the virus, and that the linkage data in general measure spatial relationships among loci.

Viral Genes. The mutations *h* and *r* occur independently of each other and also occur at different combinative loci. Different *r* mutations occur at different combinative loci. That they occur at different mutative sites is shown by the fact that the mutant *r*1*r*5 (obtained by crossing) can mutate to *r*1 (Hershey and Rotman, 1948). On the other hand, it can be shown that two *h* mutations that cannot be accumulated in the same stock by successive mutations have occurred at the same combinative locus. Finally, this locus occupies a linkage position that is independent of its mutational state. The locus, therefore, can be identified as a site of mutation, recombinative unit, and map position (Hershey and Davidson, 1951). The classical notion of the gene rests precisely on the mutual consistency of these three criteria of the unitary character of hereditary factors.

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LATENT OR DORMANT VIRAL INFECTIONS

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The purpose of this paper is to review some of our knowledge concerning latent virus infections, in the hope that such a survey of the distribution, variety and behavior of these infections may facilitate the study of the neoplastic processes in man and in domestic and laboratory animals. I shall purposely avoid giving a definition of latency, since, if a broad, ecological view of the phenomenon of survival of parasites in nature is taken, it becomes clear that the stabilization of the host-parasite relationship cannot be based only upon the multiplication of virus at the expense of destroyed cells. It is much more probable that, in many instances, a latent virus persists in its host-cell as an innocuous symbiont, with a capacity to shed virulent variants only in rare cases.¹ Thus, if the broader view is adopted, a paper on latency of viruses should really encompass all known viral infections of plants, animals and insects. This would obviously be much beyond the scope of this presentation. Therefore, following a brief discussion of the methods of detection of dormant viral infection, examples of only a few such infections will be described in detail and the others mentioned briefly.

Detection of Latent Infection. An inapparent infection may become apparent and cause signs of disease in its host whenever the delicate equilibrium between parasite and host is upset. This may be caused by a change in environmental conditions, by a disturbance of the physiologic equilibrium of the host, or by any other factor or factors of a nature as yet undetermined.

More commonly, a dormant virus infection may be detected by a careful examination of either animal or plant tissues, the cells of which are suspected to contain these viruses. If the latter is visible and morphologically identifiable, then such methods as electron microscopy can be used successfully for that purpose. In other instances, transplantation of the material into other animal or plant species, with resulting signs of infection, may constitute proof of latency. The demonstration of a specific antigen in the suspected tissue by serological tests may sometimes be the means of detecting an inapparent viral infection.

Finally, if the latent virus is able to evoke an immune reaction in its host, detection of specific antibodies and/or resistance to reinfection with the homologous agent may be considered as an indication of persistence of the virus in inapparent form in the host.

Obviously, these methods and techniques are neither exact nor exhaustive but they cannot be improved until our knowledge of the intricate mechanism of infection of living cells with viruses becomes more comprehensive. There seems to be little doubt, however, that many instances of latent infection escape notice because of the inadequacy of existing methods of detection. These reservations should be borne in mind during the ensuing discussion.

Latent Viral Infections of Mammals. In a discussion of latency of viruses, herpes simplex and rabies infections represent two contrasting problems in the extreme. Herpes simplex is a classical example of latent infection in man. The virus is dormant in the majority of human beings² and there are many flaring factors which may provoke the quiescent virus to appear. They may be listed as fever (induced or due to infection), cold, menstruation, exposure to sun, and, last but not least, emotional upset.³

Conversely, our knowledge of possible latency in rabies is very slight, and it is obvious that an answer based solely on observations in man would be inadequate. It should be pointed out, however, that circumstances surrounding the transition from the inapparent to the apparent infection in rabies may tend to incriminate certain factors. Intensive Pasteur treatment, for instance, was associated with shorter incubation periods of the disease⁴ and emotional shock was thought to precipitate an attack of rabies in an exposed individual.⁵ One case, that of a young boy bitten by a rabid dog, possibly merits detailed description. After exposure, the boy received

TABLE 1
RECHALLENGE OF HAMSTERS WITH STREET STRAIN OF RABIES VIRUS

Experiment number		Mortality ratio of hamsters exposed to street virus and injected with dilutions of antisera				
		1:2	1:8	1:32	1:64	1:128
35	Original exposure	1/10	3/10	4/10	4/10	9/10
	Rechallenge*	3/9	2/7	0/6	0/6	0/1
35a	Original exposure	0/9	1/10	6/10	5/10	8/10
	Rechallenge*	2/9	0/9	0/4	0/5	0/2

* Five months later.

the full course of antirabies vaccine treatment and showed no signs of illness. Five months later, he became ill with bacterial pneumonia and while recovering began to exhibit signs of involvement of the central nervous system and later died of rabies.⁶ Although coincidence of a prolonged incubation period cannot be ruled out in this case, the circumstantial evidence seems to demonstrate that the upset of the rabies virus-host cell equilibrium was caused by a febrile disease.

Evidence of viral latency in herpes simplex and rabies infections may be obtained in laboratory animals. Rabbits remained well, after either intramuscular or intravenous inoculation with herpes virus, but subjection to either anaphylactic or histamine shock invariably precipitated a severe encephalitis in the inoculated animals, and herpes virus could be demonstrated in the brain tissue. Characteristic pathological changes observed in the shocked animals were lacking in the control animals.^{7, 8}

In the case of rabies, injection of Syrian hamsters seemed to provide a possible clue to the mechanism of its latency. When exposure to street virus was followed 24 hours later by administration of rabies antiserum, the

lives of many animals were spared and the survival ratio was directly related to the concentration of antiserum employed. Five months later, all of the surviving animals were again challenged intramuscularly with a lethal dose of street virus and most of them were found to be immune.

It may be observed in TABLE 1 that resistance to rechallenge inoculation was inversely related to the concentration of antiserum originally employed. Lower survival ratios were observed in groups of animals which received higher concentrations of antiserum. The mechanism of immunity observed in these experiments can be explained most readily on the basis of latency of street virus infection.

Evidence that quiescence of the virus was not conditioned upon the use of antiserum is furnished by other experiments, summarized in TABLE 2. In these cases, hamsters were originally inoculated with high dilutions of street virus by parenteral route, not treated with serum, and rechallenged five months later. Again, it may be observed that in many instances,

TABLE 2
RECHALLENGE OF HAMSTERS WITH STREET STRAIN OF RABIES VIRUS

Experiment number		Mortality ratio of hamsters exposed to dilutions of street virus			
		1:640	1:2,560	1:10,240	1:40,960
30	Original exposure Rechallenge*	7/9 0/2	5/8 2/3	2/8 5/6	0/8 8/8
31	Original exposure Rechallenge*		8/9 0/1	2/9 3/7	1/9 5/8
33	Original exposure Rechallenge*	9/10 0/1	5/10 4/5	5/10 3/4	1/9 7/8

* Five months later.

though not all, resistance to rechallenge inoculation was evident. Since none of these animals showed signs of disease prior to rechallenge, inapparent infection with street virus has to be postulated.

Perhaps the most interesting phenomena of quiescence in relation to rabies are the so-called "self-sterilizing neuro-infections," a term coined by Levaditi *et al.*⁹ for a condition in which no virus can be isolated from an organ almost certainly infected. Fowl and pigeons for instance, injected intracerebrally with street virus were observed to die 42 and 18 days later, respectively; but attempts to isolate virus from the brain tissue of these animals were futile, in spite of the appearance of characteristic pathological lesions, including the presence of Negri bodies.¹⁰ The same phenomenon was observed in rabbits injected intracerebrally with a high concentration of chick-embryo-adapted rabies virus,¹¹ and, here again, attempts to demonstrate the possibly dormant virus by means of purification and fractionation of the nervous tissue were fruitless.¹¹ The supposition that the absence of the virus in the dead animals was caused by a reaction of the host, a reaction of such efficiency that it destroyed both the virus and the host, is as

good a postulate as the one that the virus, bound firmly to some tissue component, may have persisted in non-demonstrable form, but with an unimpaired ability to induce further destructive processes. It is curious to note that the above-mentioned herpetic infection of rabbits has also been described as a "self-sterilizing neuro-infection."

Other examples of latent viral infections of man are shown in TABLE 3. West Nile virus was originally isolated in 1937 from the blood of a native of Uganda who showed no signs of illness.¹² Thirteen years later, sera of three Egyptian children, who were living in the vicinity of Cairo and who did not suffer from any severe ailment, yielded a virus immunologically identical with the West Nile virus.¹³ The virus produced encephalitis in mice, hamsters and rhesus monkeys when injected intracerebrally, and produced a latent infection in chimpanzees following intracutaneous inoculation. It also caused a latent infection in man, the virus circulating in his blood for 21 consecutive days.¹⁴ Its fate thereafter remained unknown. I shall return to this problem later.

TABLE 3
EXAMPLES OF LATENCY IN VIRUS INFECTIONS OF MAN

<i>Infection</i>	<i>Recovery of latent virus</i>		<i>Longest observed period of latency (inapparent infection)</i>
	<i>Tissue</i>	<i>Method</i>	
West Nile.....	Blood	Inoculation of mice	21 days
Poliomyelitis.....	Feces	Inoculation of monkeys	5 weeks
Serum hepatitis.....	Blood	Transfusion	4 years
Psittacosis.....	Sputum	Inoculation of mice	10 years

As we now know, the inapparent type of infection with poliomyelitis virus is the rule and the paralytic case is the exception.¹⁵ In the latent infection, persistence of the virus seems to be limited to the intestinal tract and, in the example shown in TABLE 3, the virus was isolated from family associates of actual cases during a five-weeks' observation period.¹⁶ None of the virus carriers showed any signs of illness before, during, or after the period of virus excretion. Although a discussion of the flaring factors which determine the transformation of a quiescent infection into a frank disease is beyond the scope of the present paper, it might be mentioned that, in the case of poliomyelitis, it is suspected that physical activity plays an important role.^{17, 18} In addition, the practice of inoculating children with combined pertussis-diphtheria vaccines during poliomyelitis epidemics¹⁹ recently was incriminated as a factor which may predispose individuals to paralysis.

The bizarre nature of infection with serum hepatitis virus made workers suspect long ago that at least one stage of the infectious process is characterized by latency. Recently, evidence obtained by Stokes and Henle²⁰ indicates that a dormant stage of infection is not necessarily preceded or followed by a period of frank symptoms. TABLE 3 summarizes the clinical history of two cases. The first is that of a chronic alcoholic who trans-

mitted serum hepatitis to four patients during two years as a professional blood donor, and was found to be a carrier after another two-year period. The results of his liver function tests were not entirely normal, which is explainable perhaps by his alcoholism, but he had neither signs nor history of jaundice or any other disease.

The other case is that of a woman whose baby developed jaundice several months after birth. The mother's blood was found to transmit serum hepatitis to volunteers at that time and again two years later. Clinical tests revealed no abnormal liver function and she had no history of jaundice.

Latency has perhaps never played such an important, if not preponderant, role in any other viral disease as it has in psittacosis. In the words of Meyer,²¹ "These latent, inapparent infections represent the corner-stone of the entire psittacosis and ornithosis problem."

It may be observed in TABLE 3 that a record of duration of the carrier state in a viral disease has been established in the case of one unhappy individual who contracted a severe psittacosis infection and in whose blood and sputum, during the acute stage, the causative agent was demonstrated. Following his recovery, and during the ensuing ten years, he shed the virus in his respiratory secretion and, although he often had paroxysms of productive coughing, the patient remained well.²² Another series of five cases of latent infection with psittacosis virus was described in Vienna,²³ in which none of the victims had clinically recognizable signs of the disease. In one of the latter cases, virus was demonstrated in the blood 72 days, and, in the sputum, 75 days after the initial virus isolation.²³

Viral respiratory infections of man, other than psittacosis, occur in a latent form, as, for example, inapparent infection with influenza.²⁴ One should also mention that the instillation of normal yolk sac suspension into the nasal cavity has been shown to activate an apparently latent infection with the common cold virus.²⁵

The criteria of latency become more uncertain in viral infections of animals. It would be obviously impossible, for example, to attempt to make a clinical diagnosis based on the number of cases of headache, dizziness, nausea, general malaise or fever among members of a mouse colony who were found to harbor and/or shed one or another viral agent. Keeping these reservations in mind, the pattern of dormant virus infections in man is reproduced in animals and the knowledge concerning these latent viruses in animals complements information on human disease in some instances.

Canine infectious hepatitis (TABLE 4) is a disease characterized by a bizarre course, causing marked changes in the liver of the affected animals. The saliva of infected dogs was found to contain the virus during the febrile period of infection. Recovered animals, however, were found to shed the virus in urine for 161 days following recovery. The virus apparently persisted in quiescent form in the kidneys, causing a focal interstitial nephritis.²⁶

The characteristic features of mouse encephalomyelitis parallel those of human poliomyelitis. The virus was found to be present in the intestinal tract of normal mice which probably became infected during the first day of life.²⁷ Paralysis was observed among the virus-carrying animals only

occasionally and no explanation could be found for the flaring factors or "jolts" which transform a symptomless carrier into a paralytic case. Similarly, lymphocytic choriomeningitis virus was found to infect 100 per cent of the mice in some colonies without causing signs of illness, and virus was demonstrated in the blood, urine and the nasal washings for at least four to five months afterwards.²⁸ The infection was acquired *in utero* and persisted in latent form throughout the life of the mouse. The virus was isolated from the semen of virus-carrying males, and house mice were found to be carriers of dormant lymphocytic choriomeningitis virus as frequently as the white mice.²⁹

Psittacosis virus appears onstage again, this time as a latent parasite of birds. Numerous other species of the Class *Aves*, in addition to parakeets, are known to carry inapparent infections with this virus. However, the

TABLE 4
EXAMPLES OF LATENCY IN VIRUS INFECTIONS OF ANIMALS

Infection	Host species	Recovery of latent virus		Longest observed period of latency
		Tissue	Method	
Infectious hepatitis (canine)	Dog	Urine	Inoculation of dogs	161 days
Mouse encephalomyelitis	Mouse	Feces	Inoculation of mice	53 days or more
Lymphocytic choriomeningitis	Mouse	Feces, Urine, Semen, Nasal Secretion	Inoculation of mice	Life-long (if infected <i>in utero</i>)
Psittacosis	Parakeet	Spleen, Intestinal content, Nasal secretion, Kidneys	Inoculation of mice	385 days

parakeet is given as the example, since some of the flaring factors which disturb the host-virus equilibrium in favor of manifest infection are known. These are: crowding the birds in unsanitary cages, improper feeding, rapid changes in temperature, and other unfavorable environmental conditions.²¹

In TABLE 5, data are summarized for two viral sheep infections, prevalent in England, in which latency plays a very prominent part. Scrapie, in its manifest form, is characterized by an intense and progressive pruritus, progressive asthenia, and locomotor incoordination. Since it is not possible to transmit the disease by artificial inoculation in the natural host, it has been postulated that infected rams may transmit the infection to their offspring, which succumb to the disease at the age of two years. The ewes mated by the "scrapie rams" remain unaffected.³⁰ Recently, using brain and cord tissue of infected animals as a source of virus, transmission experiments by intracerebral inoculation of sheep were successful.³¹ The incubation period, however, was found to vary from six months to two years and only a fraction

of the inoculated animals showed signs of disease. The virus remained quiescent in the remaining sheep.

Ovine enzootic abortion is another example *par excellence* of latent viral infection. The dormant virus is known to belong in the psittacosis group of viruses.³² Mating and the resulting pregnancy seem to make the infection manifest since the infected ewes usually, but not always, abort. The extent of latent infection in young lambs can be demonstrated by subinoculation of their tissues in other sheep or in the yolk sac of developing chick embryos. As seen in TABLE 5, the virus can be recovered from symptomless animals as late as eight months after exposure.³³

Numerous other examples of latent viral infections of animals may be cited: salivary gland virus in rodents,³⁴ influenza virus in swine,³⁵ pseudorabies virus in swine,³⁶ infectious anemia in horses,³⁷ virus III in rabbits,³⁸ and, also, the Snotseikte virus in a latent form in the wildebeest, and African swine fever in wart hogs.³⁹

TABLE 5
EXAMPLES OF LATENCY IN VIRUS INFECTIONS OF SHEEP

Infection	Recovery of latent virus		Longest observed period of latency
	Tissue	Method	
Scrapie	CNS Sperm (?)	Inoculation of sheep Transmission to offspring (?)	2 years or more 2 years or more
Ovine enzootic abortion	Fetal membranes Spleen Lymph nodes	Abortion Microscopic smears Yolk sac inoculation	8 mos. or more

Latent Infection of Insect Vectors with Plant and Animal Viruses. There are many examples of symbiotic persistence of viruses in their insect carriers. The clover club-leaf virus was transmitted through 21 generations of leafhoppers during five consecutive years without causing any apparent adverse effect upon the vector in which it multiplied.⁴⁰

Two cases of transovarian transmission of latent virus may also be cited. St. Louis encephalitis virus was found in nymphs hatched from eggs laid by an infected bird mite *Dermanyssus gallinae*,⁴¹ and Colorado tick fever virus was passed transovarially to the next generation of adult wood ticks *Dermacentor andersoni*.⁴²

So far, in spite of the presence of the plant and animal viruses in the insect vectors, no one has as yet been able to detect a deleterious effect of these viruses in their insect carriers, even in those cases where the viruses are known to multiply in the host.⁴³

*Latent infection by plant viruses.** A classical example of latent infection in the field of plant viruses is furnished by the potato X virus, which was

* The author is indebted to Dr. L. M. Black, of the Brooklyn Botanic Garden, for his invaluable counsel in the preparation of this section.

found to remain completely quiescent in certain potato varieties. The incidence of inapparent infection was so great that, at one time, it was said that all American potato varieties were infected with the virus, yet its presence was entirely unnoticed. It has been shown, paradoxically, that potato plants, inapparently infected with certain mild strains of potato X virus, outgrow and outyield healthy controls.⁴⁴ The presence of the latent virus was originally determined by subinoculation into other susceptible species of plants.⁴⁵ Certain potato varieties occasionally suffer from severe disease as a result of infection with certain strains of potato X virus.⁴⁶

Apparently healthy sugar beets and marigolds collected at random from different localities in Great Britain were found to be infected with a latent virus, the presence of which could be demonstrated by the inoculation of cowpeas.⁴⁷

Apart from the entirely symptomless, latent type of virus infection, the phenomenon of "acquired immunity" in plant diseases is associated with a

TABLE 6
EFFECT OF ENVIRONMENT UPON LATENCY OF CERTAIN PLANT VIRUSES

<i>Virus</i>	<i>Temperature (degrees in centigrade)</i>	<i>Latent or apparent infection</i>
Potato mosaic	>24 <20	Latent Apparent
Potato yellow dwarf	<16 24-28	Latent Apparent
Cabbage black ring	16 28	Latent Apparent
Tobacco mosaic	>35 or <7-10 15-20	Latent Apparent

virus-carrier state, which follows the acute stage of the infectious process but then remains dormant in the recovered plant. Again, there are many examples, but perhaps the most outstanding is the one originally described by Wingard,⁴⁸ who made his observation on tobacco plants (*Nicotiana tabacum*), which recover completely from infection with tobacco ring-spot virus. Although these plants appear healthy, the virus is still present and can be demonstrated by inoculation of healthy non-carrier plants. The same mechanism of so-called acquired immunity was described in connection with tobacco-streak virus,⁴⁹ i.e., the presence of latent infection precludes reinfection by homologous virus.

Perhaps the most interesting phenomena in connection with dormant viral infections of plants are the effects of environment upon latency. Because of the nature of the problem, it has been much better studied in the field of plant viruses than animal viruses and it may be instructive to discuss the so-called "masked" plant viruses briefly.

In TABLE 6, examples are given of virus diseases of plants which may cause either a masked or an apparent infection, depending on the temperature of

the environment. It may be observed that symptoms of infection with potato mosaic virus are suppressed at temperatures above 24°C., whereas the virus becomes manifest at temperatures below 20°C.⁵⁰ A completely reverse situation exists in the case of potato yellow dwarf virus, which will remain masked at temperatures below 16°C., but which will flare up at temperatures of 24–28°C.⁵¹ The same conditions were observed in the case of cabbage black ring virus.⁵² Holmes' masked strain of tobacco-mosaic virus was isolated from infected stems treated for various times at 34–35°C.⁵³ Since then, it has also been discovered that tobacco mosaic virus remains in a masked form at temperatures below 7–10°C.⁵⁴

Occurrence of Viruses in the Course of Propagation of Tumor Tissue in Mice and Eggs. The occurrence of viruses in mice, hamsters and eggs during passages of several types of neoplastic tissue, which I will now describe, will sound as unbelievable to you as it does to me. It will be presented, however, for the sake of argument and with the hope that other laboratories will try their hand at similar experiments.

During the past three years, many attempts were made to propagate transplantable mouse tumors in the central nervous tissue of mice following the technique described years ago by Shirai.⁵⁵ In almost all instances, tumors normally propagated by the subcutaneous route were grown in the brain tissue of mice. The mice inoculated intracerebrally usually showed signs of involvement of the central nervous system on the 9th to 14th day after implantation and, on autopsy, growing neoplasms were demonstrated by histopathological examination, and on bioassay. Some of the tumors underwent passage by this technique through 50 to 80 generations. In certain instances, however, in the course of subjecting some of the tumors to passage, mice became sick three to five days after inoculation with brain tissue material stemming from the preceding passage. Careful investigation of the resulting passage indicated that a filterable agent was causing the sickness of the animals,⁵⁶ and the study was extended in order to learn more about the nature of the phenomenon.

In TABLE 7 data are summarized covering the isolation of the viruses recovered in the course of these tumor studies.* The Ridgeway osteogenic sarcoma obtained from an outside laboratory was transplanted subcutaneously in AKM mice through eight passages, and tumor suspensions then were injected intracerebrally into Swiss albino mice. These animals became sick four days later, and the recovered virus was tentatively identified, on the basis of preliminary results of neutralization tests, as being related to Ilheus virus. In order to exclude the possibility that a latent virus might have been picked up from the host mice during the eight passages in the laboratory, another attempt at intracerebral passage of neoplastic tissue was made, using fresh tumors immediately upon receiving them from the outside laboratory. These tumors were injected directly into mice by the intracerebral route. Again, the same virus was isolated.

In the case of a Wagner osteogenic sarcoma, filterable agents were isolated

* The author expresses his grateful appreciation to Dr. Alice E. Moore, of the Sloan-Kettering Institute for Cancer Research, for the mice-bearing tumors; and to Dr. Stanley Stellar, of the Department of Neurosurgery, New York School of Medicine, for the human tumors.

after two or three subcutaneous transplants, followed by one or two intracerebral passages. At this point, it became quite obvious that a plausible explanation of this phenomenon could be that a latent viral infection persisted in the mice of our laboratory, and that intracerebral inoculation of such a fast growing tissue as neoplasm made the infection manifest. In the next attempt, therefore, a suspension of Wagner osteogenic sarcoma propagated through four mouse passages by subcutaneous transfer was inoculated into the yolk sac of six-day-old chick embryos. The embryos were incubated at 37°C. and harvested three days later. A ten per cent embryonic suspension was injected intracerebrally into mice and underwent passage

TABLE 7
OCCURRENCE OF VIRUSES DURING PASSAGES OF TRANSPLANTABLE
MOUSE TUMORS

Tumor		Isolation of virus			
Type	Number of subcutaneous transplants in mice*	Host species	Number of passages preceding isolation		
			Mouse i.c.	Hamster i.c.	Chick embryo y.s.
Ridgeway osteogenic sarcoma	8	Mouse	1		
	None	Mouse	1		
Wagner osteogenic sarcoma	2	Mouse	2		
	3	Mouse	1		
	4	Chick embryo	None		10
Patterson lymphosarcoma	13	Mouse	6 & 5		
	13	Chick embryo	6		5
	19	Chick embryo	None		5
Spontaneous† mouse tumor	None	Chick embryo	None		5
MC-1	Numerous	Hamster	27	7	

* At Lederle Laboratories.

† Mammary adenocarcinoma.

into the yolk sac of six-day-old embryos. No signs of disease were observed in either the embryos or the mice injected with embryonic tissue, representing the first through the ninth egg passages. Embryos injected with the tenth embryo passage, however, died 48 to 72 hours after inoculation, and the inoculation of an emulsion of these into mice yielded a virus identical with that isolated by means of mouse passage. This virus, on the basis of preliminary neutralization tests, was found to be related to the West Nile, Japanese B, St. Louis, Ilheus group of viruses.

Similar results were obtained with the Patterson lymphosarcoma, except that in two passage series, the tumor was transplanted for 13 and 18 passages by the subcutaneous route in the AKM strain of mice before a filterable agent could be isolated in eggs or in mice injected intracerebrally. When

isolated, this agent, on the basis of preliminary neutralization tests, was found to be identical with the one isolated in the preceding experiments with the Wagner osteogenic sarcoma. It may be of interest to note that, in the case of the Patterson lymphosarcoma, with the 13th and the 18th passage inocula as the original material, it took exactly the same number of passages (three mouse brain and five chick embryo) before the presence of a virus was demonstrated.

A spontaneous mammary adenocarcinoma observed in a normal mouse was next removed and a suspension of it injected into the yolk sac of fertile eggs. Five "blind" passages yielded a virus lethal to embryos and mice, and identical with the strains isolated in previous experiments. In order to avoid intracerebral mouse passage in propagating neoplastic tissue, a suspension of a methyl-cholanthrene tumor (MC-1), maintained by subcutaneous transplantation in Swiss albino mice, was injected intracerebrally into Syrian hamsters. Sixty days later, the inoculated animals were found paralyzed and the brain tumors present were subinoculated into other groups of hamsters. At the seventh consecutive passage in hamsters, the animals became sick four days after inoculation, and a filterable agent related to West Nile virus was isolated.

In all of these experiments, brain passage of the tumors was essential for isolation of the virus.

In order to investigate further the significance of these isolations, a broader study was undertaken and a number of attempts were made to adapt human tumor tissue to mouse brain passage. Briefly, the technique of these experiments consisted of the following: A ten per cent suspension of the tumor was injected intracerebrally into 10- to 12-day-old Swiss albino mice which were obtained from a breeding stock free of mouse encephalomyelitis virus. If no signs of central nervous system involvement were observed, the animals were sacrificed six to eight days after inoculation and their brain tissue made into a suspension and passaged into another group of 10- to 12-day-old mice of the same stock. In all, 27 human neoplasms were used in these experiments, and, as seen in TABLE 8, a filterable agent became manifest in only three instances. In the remaining 24 cases,* no signs of sickness were observed in the inoculated mice, and no isolation was made in subsequent blind passages. It may be seen from TABLE 8 that mice injected with the McK and R tumors sickened four to seven days after primary inoculation and brain passages were made to other mice, while a single blind passage was made in the case of the J tumor. In all three instances, however, a filterable agent was isolated. The original McK tumor was kept frozen for 24 days at $-70^{\circ}\text{C}.$, after which a second attempt again resulted in isolation of the same agent. Finally, one year later, the same McK tumor was again removed from the freezer and a ten per cent suspension injected into the yolk sac of six-day-old embryos. Half of the inoculated embryos were found dead 72 hours later, and the isolated agent was identified in eggs as being related to the West Nile group of viruses. It may be added that

* Adenocarcinoma of the rectum—two cases; ependymoma—one case; adenoma of the rectum—one case; meningioma—three cases; glioblastoma multiforme—four cases; astrocytoma—one case; glioma—three cases; acoustic neuroma—one case; adenocarcinoma of the breast—three cases; sarcoma of the thorax—one case; as yet unidentified brain tumors—four cases.

the J virus was found to be non-pathogenic for adult mice during the first 20 passages in baby mice. Subsequently, it became possible to adapt the virus to adult mice, and the resulting agent again seemed to be related to the West Nile, Japanese B, St. Louis, Ilheus group of viruses, as indicated by the preliminary results of neutralization tests.

The McK virus was adapted to adult mice after six passages in baby mice, and the resulting agent was found to be identical with the virus which became manifest during attempts to passage the same tumor tissue in developing chick embryo. It is of certain interest to note that antisera which seemed to neutralize the J and the McK viruses after adaptation to adult mice, seemed to have no neutralizing capacity against the same viruses when propagated in baby mice.

These phenomena require some explanation. The probability of a latent viral infection present only in mice cannot be considered an adequate explanation, since in one instance the virus became manifest during passages

TABLE 8
OCCURRENCE OF VIRUSES DURING ATTEMPTS TO ADAPT HUMAN
TUMORS TO MOUSE BRAIN

<i>Tumor</i>	<i>Isolation of virus</i>			
	<i>Host species</i>	<i>Age of host (days)</i>	<i>Number of passages</i>	<i>Mortality ratio</i>
Adenocarcinoma of rectum J	Mouse	10-12	2	7/7
Adenocarcinoma of rectum McK	Mouse	10-12	1	3/4
	Mouse	10-12	1	1/8
	Chick embryo	6	1	4/8
Metastatic carcinoma of brain R	Mouse	10-12	1	6/6

in chick embryos without any intervening passage in mouse brain. The possibility cannot be excluded that both chick embryos and mice are latent virus carriers and that the presence of virus becomes manifest only after the injection of tumor tissue into these hosts; but this perhaps seems to be too far-fetched.

One may theorize that a symbiant, perhaps related to West Nile virus which has been shown to cause latent infection in man, is present in the body and is drawn by fast growing tumor cells, but the absence of antibody response in the two individuals whose neoplastic tissue was processed in the laboratory does not seem to give adequate support to this theory.

Contamination with virus of material processed in the laboratory may offer the best possible explanation for the phenomena, even though in experiments on many other unrelated projects blind passages in the same strains of mice resulted in no such isolation of virus.

While the subject under discussion is latent or dormant viral infections, there seems to be only a remote possibility that in the above cases viruses in quiescent form were present in the original tumors. The problem is still

wide open for further study. It has been brought up at this meeting not because there is a satisfactory explanation of the phenomena but in the hope that it will encourage other laboratory workers to pursue similar types of study. The results of these may perhaps throw some additional light on the properties and characteristics of a group of viruses which, on the basis of preliminary results of neutralization tests, were found to be related to the West Nile, Japanese B, St. Louis and Ilheus group of viruses, and which perhaps play a more significant role than originally suspected as laboratory contaminants* rather than as latent or dormant infections.

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*Laboratory contamination with Japanese B encephalitis virus has been described at least in one case in the past.⁵⁷

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STUDIES ON THE COMBINED EFFECTS OF FOWL POX VIRUS AND METHYLCHOLANTHRENE IN CHICKENS*

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The present investigation deals with the problem of the dual role of certain viruses that can manifest themselves either as cell stimulants or cell destroyers, depending on environmental conditions, of which the age factor is of primary importance.[†] An example is the fibroma virus of the rabbit, which induces an acute, lethal and largely destructive disease in the newborn if injected in large amounts, although it may induce local and generalized fibromas if injected in small amounts or after storage. In the adult, this virus induces only mild local tumors,³ irrespective of the amount of the inoculum.

Other examples are the viruses of avian tumors, which induce sarcomas in the adult host, but cause generalized destructive lesions in viscera and central nervous systems in the embryo or the newborn.⁴⁻⁷

Therefore, it appears that although such viruses belong to a category separate from that of ordinary viruses when behaving as neoplastic agents, they belong to the category of ordinary viruses when behaving as cell destroyers. These viruses, whether neoplastic or cell-destroying, are nevertheless, generally accepted as "neoplastic" only when they are similar to ordinary viruses in composition, antigenicity, ways of transmission, capacity of variation, capacity of masking and unmasking, and conditioning by genetic and environmental factors. The problem we are trying to elucidate, therefore, is whether such ordinary viruses, accepted as eventually being "destructive" only, are also endowed with a neoplastic capacity that can manifest itself in a propitious ground, just as the "neoplastic" viruses of avian tumors are, as a rule, only neoplastic in the propitious ground of a grown host. As an example in favor of our hypothesis one could cite, among others, the case of the sheep pox virus, which, induces a mild papillomatosis in the adult, although essentially destructive and often lethal in the young host.^{8, 9}

Our first task, therefore, was to render the ground "propitious," and we made use of the so-called carcinogens for this purpose, while approaching the problem in two ways: first, by searching for the appearance, in the skin lesions caused by the carcinogen (preferably before reaching malignancy) of ordinary viruses presumably latent in the host; and second, by the injection of a given ordinary virus, not latent in the host, after this host had been prepared by the carcinogen.

The present preliminary report is concerned with the results obtained so far in chickens and pigeons submitted to repeated paintings with a methyl-

* This investigation was supported by grants from the Jane Coffin Childs Fund for Medical Research, The American Cancer Society, and the National Cancer Institute of the National Institutes of Health of the United States Public Health Service.

[†] The topic was extensively discussed in two recently published reviews.^(1, 2)

cholanthrene solution. The basic findings are that such treatment first causes an ordinary pox virus, latent in the birds, to become active; and that this virus may be recovered in the variety of neoplastic lesions that later develop on continuation of treatment with the carcinogen.

Materials and Methods. The main investigation, on chickens, carried out in the course of three years, consisted of the following experiments: 51 A, started August, 1947, 28 test birds; 51 B, started September, 1948: 25 test and 40 control birds; 51 C, started October, 1948: 39 test and 47 control birds; and 51 E, started January, 1950: 120 test and 28 control birds.

Experiment 51 E is divided into four groups: (a) 1, (a) 2, (b) 1, and (b) 2, each group containing 30 test chicks. Therefore, of the total of 327 birds, 212 served as test and 115 as controls. The chickens were of the Plymouth Rock variety, aged either 30 days, in experiments 51 A and B; or three days, in experiments 51 C and E, when paintings were started. They were obtained from a local firm which hatches eggs collected from several New England states. The methylcholanthrene was used in a 0.3 per cent solution in ether and mineral oil.

The test chicks were painted in the right side of the breast either twice or six times a week, so that all of them initially received 82 paintings in either $3\frac{1}{2}$, or in 8 to 9 months. More series of paintings were given subsequently to many of the birds. The feathers were plucked from the treated skin before each painting. For reasons to be stated later, testosterone (generally from 12 to 24 mg.) was injected, either coinciding with the paintings or independently from them.

The control birds were set up to investigate the possible development of fowl pox infection in the absence of treatment with methylcholanthrene. Accordingly, 73 birds were left untreated although their feathers were plucked, as in the test chickens, while 27 chickens from experiment 51 B, and 15 from experiment 51 C, were injected with either 12 or 24 mg. of testosterone. All of the controls were kept in cages in close contact with the test animals.

Biopsies, for histological studies and investigation of the presence of virus, were taken from lesions developed in test and control birds and also from grossly normal skin. A piece of tissue a few mm. in diameter was excised. Half of it was fixed in 10 per cent formalin. The other half was ground with sand and approximately five volumes of saline, and the supernatant fluid was rubbed on the scarified dermal areas of three chicks. A similar procedure was followed when dealing with the viscera of chickens that died.

Data on Survival of the Experimental Birds. The test chickens (excepting 40 from experiment 51 E, which were killed at the age of three months) were followed until their death, which seemed to be accelerated by treatment with the carcinogen. 68 birds died during the course of the paintings. In the remaining 104, an average of 55 per cent survived at the end of nine months (the extreme values were 35 per cent and 75 per cent); and 30 per cent survived at the end of 15 months (the extreme values were 0 per cent and 57 per cent).* This is to be compared with 71 per cent survival in 59

* In experiment 51 E, however, in which the chickens generally received less paintings than in other experiments, 20 birds are still alive, almost two years after the experiment was started.

untreated, control chickens from experiments 51 C and 51 E that were kept for periods 7 to 9 months.

Aside from the skin lesions to be described later, the birds that died almost constantly exhibited extensive degenerative lesions in kidneys and liver, which demonstrates that treatment with the carcinogen resulted in systemic, as well as local effects.

The Development of Gross Inflammatory and Neoplastic Lesions in the Skin Treated with Methycholanthrene. The activation of the latent fowl pox infection by the carcinogen was manifested by the development of discrete lesions, strictly limited to the painted area, at the stage where the skin began to show the characteristic thickening as currently induced by methylcholanthrene. Generalized large lesions in combs, wattles and feet, which usually develop in epidemic pox, were never observed.

These early skin lesions were observed in ratios varying roughly from 10 per cent to 70 per cent of the birds in the different experiments. A few of the lesions appeared about four weeks after the paintings were initiated, while more developed after another 3 to 5 weeks. The lesions measured from a fraction of one mm to perhaps two mm. They were generally dry, crusty, and slightly raised, although in a few cases they tended to resemble the characteristic florid pox resulting from experimental inoculation (FIGURES 1-5). It is difficult to make statements about the further evolution of these lesions as grossly observed. Some undoubtedly regressed, while others apparently changed into, or became merged, with the more obvious proliferative lesions of later appearance.

The thickness of the skin increased further on continuation of the methylcholanthrene paintings. Papillomas could be discerned in all chickens by the second or third month and, later, some of these lesions changed into squamous cell carcinomas (FIGURES 8, 9, 12, and 13). A unique feature in the reaction of fowl's skin to methylcholanthrene was the development of a large number of angiomas (FIGURES 6 and 7). Inflammation frequently was observed to accompany all of the above lesions. Most of the lesions occurred in the right, painted side of the breast. The left side was involved only occasionally.

The data indicating the incidence of neoplasia in the treated chickens are given in TABLE 1. For a better understanding of the results, the following information is offered:

(a) Only chickens that lived longer than 8 months are included in the table.

(b) In experiments 51 A, 51 E (b) 1, and 51 E (b) 2, the 82 paintings were given in $8\frac{1}{2}$ or 9 months without interruption at the rate of two paintings a week.

(c) In the remainder of the experiments, the first series of 82 paintings was given in $3\frac{1}{4}$ months at the rate of six paintings a week. The additional paintings were started after intervals of $4\frac{3}{4}$ months in 51 B, $2\frac{3}{4}$ months in 51 C, and six months in 51 E (a) 1 and (a) 2.

(d) In the latter experiments, the number of paintings is expressed in averages. The minimum number of paintings was 82 in all cases, and the

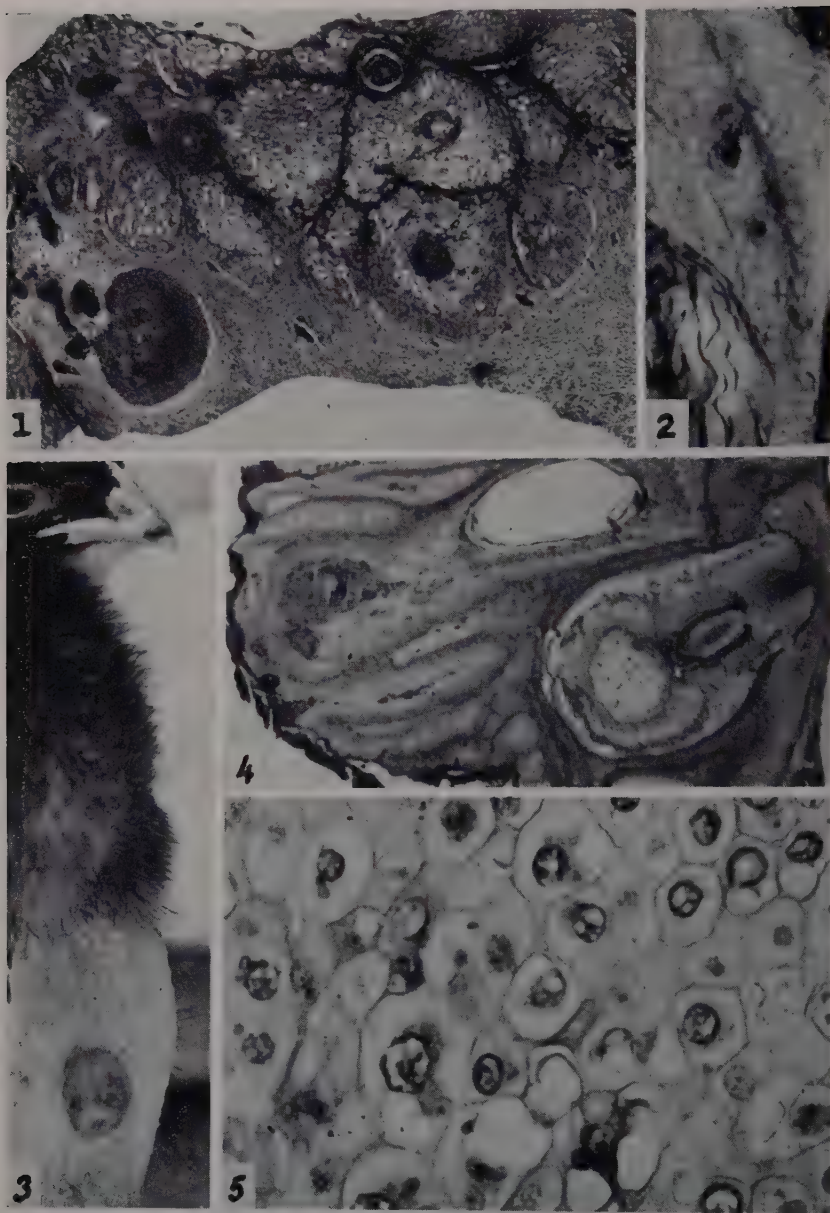


FIGURE 1. Florid fowl pox lesion, in a chicken of experiment 51 B, after the bird received 28 methylcholanthrene paintings in 60 days. Note the typical masses of pox tissue in a fibrous, hyperplastic chorion, and a nodule of lymphoid-like cells at the lower left. Magnification: 52X.

FIGURE 2. Type of fowl pox lesion, nine days old, commonly developing in chicks scarified with extracts of lesions from the methylcholanthrene painted chickens. Note predominant involvement of feather follicles and early necrosis.

FIGURE 3. A rarer type of fowl pox lesion, six days old, developing in chicks scarified as above.

FIGURES 4 AND 5. Microscopic images of fowl pox lesions at the ninth day. Note blurred appearance and huge, characteristic Bollinger inclusion bodies. Magnification: 30X and 460X.

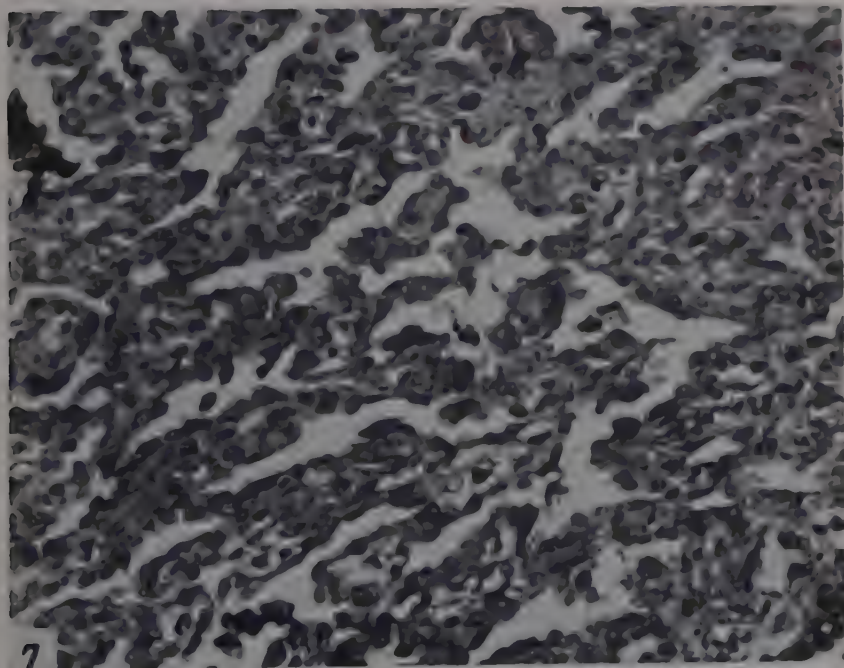


FIGURE 6. Angioma observed in one of 11 chickens kept at 31°C after the pathogen with methylcholanthrene in 11 months.

FIGURE 7. Microscopic image of the angioma. Magnification: $500\times$.

maximum was 142 for experiments 51 C (a) 1 and (a) 2, and 224 for experiments 51 B and 51 C.

The development of cancers depended on several factors. The number of paintings and the duration of the treatment seemed to be the most important. For example, in the chickens of experiments 51 E (b) 1 and (b) 2, which received 82 paintings in nine months, cancers began to appear by the sixth month, and attained their maximum incidences of 66 per cent and 41 per cent by the twelfth month. On the other hand, in the chickens from experiments 51 E (a) 1 and (a) 2, which received the same number of paintings in $3\frac{1}{4}$ months, no cancers and 16 per cent of cancers, respectively, had developed by the twelfth month. When the birds in the latter groups

TABLE 1
INCIDENCE OF DERMAL NEOPLASTIC LESIONS AFTER TREATMENT OF THE CHICKEN
SKIN BY METHYLCHOLANTHRENE
(RESULTS ON BIRDS THAT LIVED LONGER THAN EIGHT MONTHS)

<i>Experiment</i>	<i>Number of chickens</i>	<i>Number of methylcholanthrene paintings</i>	<i>Papil- lomas</i>	<i>Angiomas</i>	<i>Squamous cell car- cinomas</i>
51 A	13	82 in $8\frac{1}{2}$ mos.	13 (100%)	2 (15.4%)	6 (46.2%)
51 B	18	137 in 14 mos.* (average)	18 (100%)	3 (16.7%)	1 (5.6%)
51 C	17	155 in 13 mos.* (average)	17 (100%)	11 (64.7%)	4 (23.5%)
51 E(a) 1	16	131 in 14 mos.* (average)	16 (100%)	13 (81.3%)	11 (68.8%)
51 E(a) 2	13	123 in 14 mos.* (average)	13 (100%)	11 (84.6%)	9 (69.2%)
51 E(b) 1	15	82 in 9 mos.	15 (100%)	12 (80.0%)	10 (66.7%)
51 E(b) 2	12	82 in 9 mos.	12 (100%)	7 (58.3%)	5 (41.7%)
Total	104		104 (100%)	59 (56.7%)	46 (44.2%)

* 82 of the paintings given in $3\frac{1}{4}$ mos.

were given 50 to 60 additional paintings, however, the incidence of cancer reached 68 per cent and 69 per cent in the following six months.

In the earlier stages of the experiment, these cancers were very much dependent on treatment for growing or even maintaining themselves in the hosts. On cessation of the paintings, minute and discrete cancers, such as those developing during the first months, and even larger growths of later appearance, regressed quite readily (FIGURES 10 and 11). However, in six of the 24 birds of experiment 51 E (that survived almost two years and had not received any treatment for the last ten months), large cancers developed from the base of rare papillomas or horns that had remained in an inactive state since the previous series of treatments. In three of the birds that died of their tumors, widespread metastases were observed in the viscera, an event never observed with cancers that developed earlier.

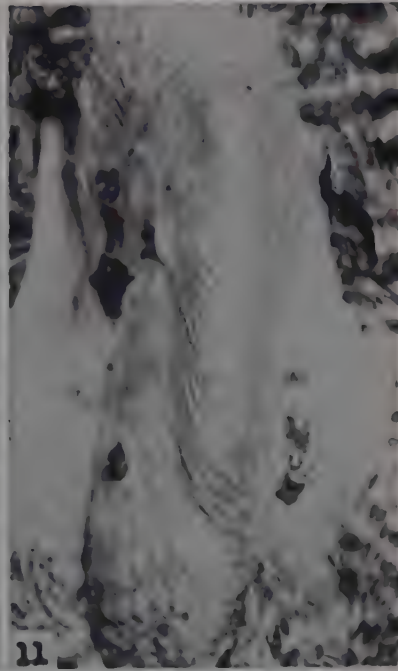
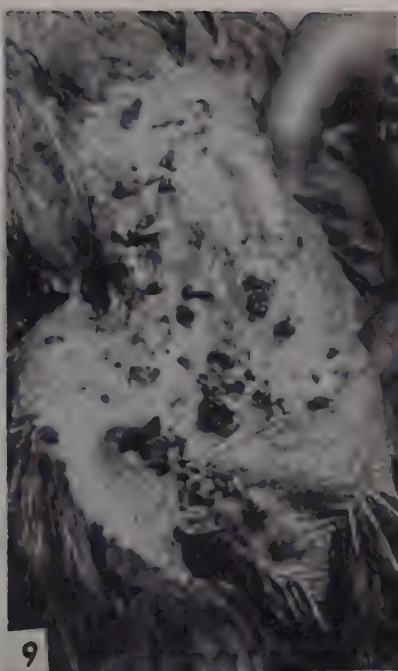
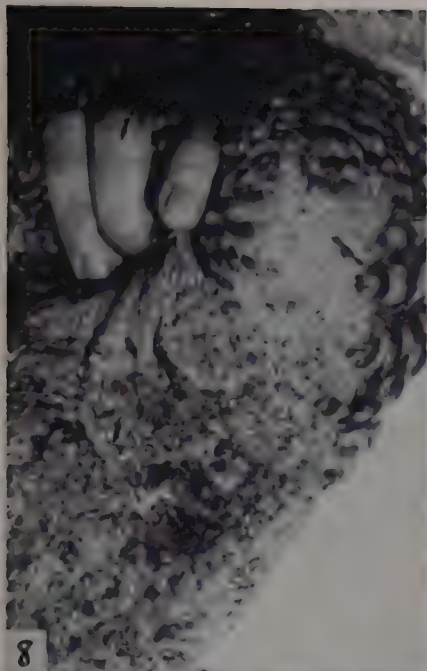


FIGURE 8. An example of resistance to carcinogenesis. The chicken, from experiment 51 C, received a total of 191 paintings in two treatments spaced over a period of 12 months. The picture was taken at the 180th painting, the bird dying shortly afterwards. The lesions were of a papillomatous and inflammatory type, cancers or angiomas never developing.

FIGURE 9. A chicken from experiment 51 E (b) 2 at the 78th day of the 82 paintings given in nine months, showing numerous papillomas and horns. The bird died at the 20th month of the experiment, with large skin cancers developing from some of the above lesions, and widespread metastases.

FIGURE 10. Squamous cell carcinoma developing in a chicken from experiment 51 A two months after having received the last of 82 paintings with methylcholanthrene together with 12 mg. of testosterone, given in a period of 8 1/2 months.

FIGURE 11. The same chicken of FIGURE 10, 2 1/2 months later, showing almost total regression of the tumor.

Transplantation, at least into a first generation of chicks, has been achieved with one of the tumors. A typical trait of these cancers, both primary or metastatic, was their pronounced tendency to undergo necrosis.

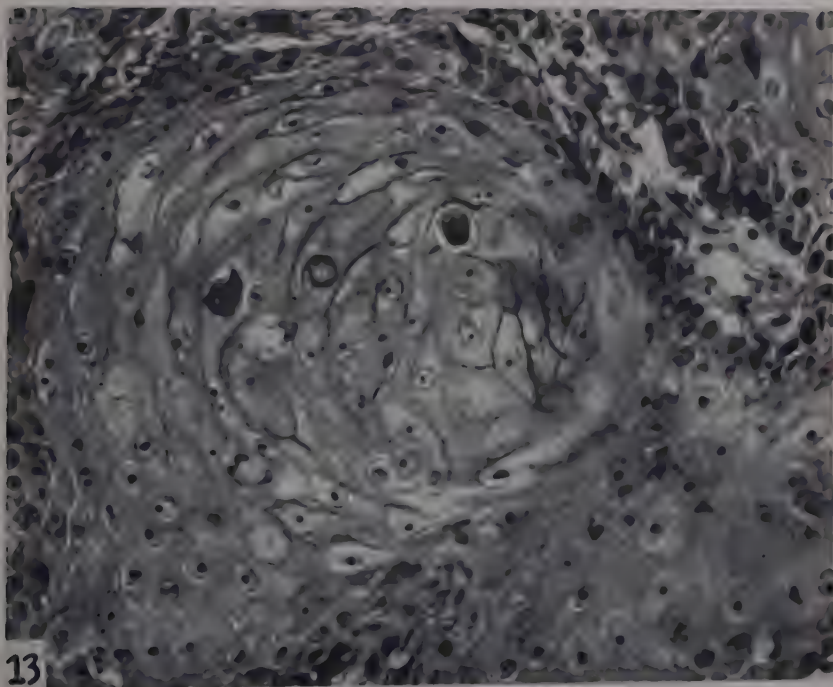
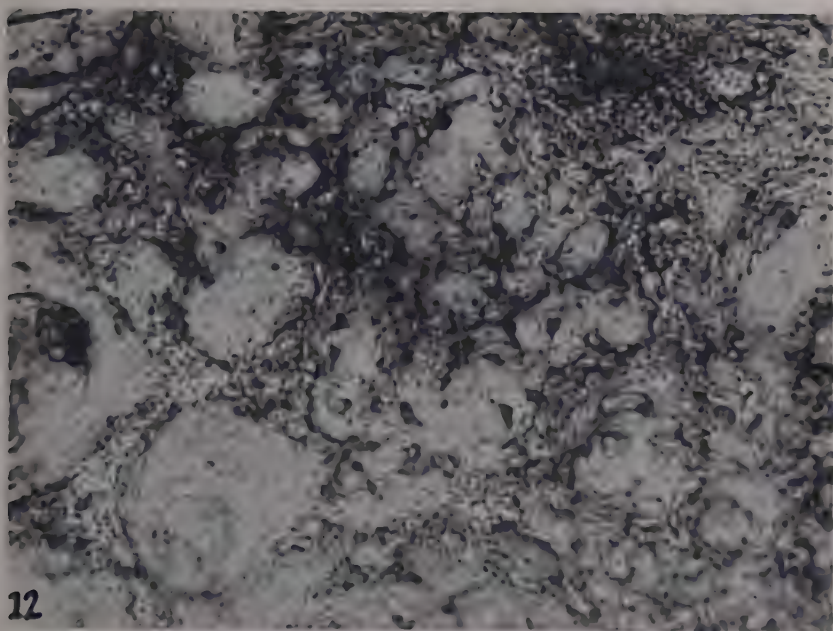
From data in TABLE 1, with the exception of experiment 51 A, it is seen that the incidence of angiomas was above that of squamous cell carcinomas. Whether conditions leading to the growth and persistence of angiomas are exactly the same as in the case of cancers cannot be said at the present time. Some angiomas persisted for many months in the absence of treatment.

Possible Effect of Hormones on Development of Neoplasia. With the view of ascertaining whether hormonal influences would modify the effect of methylcholanthrene in any direction, many of the test chickens were injected with testosterone, generally in amounts of 12 or 24 mg., sometimes in much larger amounts, given intermittently over long periods. On some occasions, it was observed that treatment with the hormone was followed by enhanced growth of tumors already present, or by the appearance of new tumors. The vagaries in the development of neoplastic lesions, however, and the difficulties of dissociating the effects of the hormone from those of the carcinogen preclude final conclusions before a close analysis of the available results is carried out or more experiments are performed. Estrone was also administered to some of our birds without definite effects.

Microscopic Observations on the Skin Lesions. Some of the early lesions which grossly suggested florid pox showed, histologically, the pathognomonic fowl pox cells with the revealing inclusion bodies. The pox tissue was observed imbedded in a hyperplastic chorion infiltrated by lymphoid cells, the latter often characteristically arranged in nodules (FIGURE 1). More frequently, however, the lesions consisted of an hyperplastic epithelium and the thickened and infiltrated chorion.

From the microscopic study of these lesions, compared to lesions that later developed on continuation of the treatment, the impression was gained that there was a gradual transition from the acute or sub-acute early pox to the squamous cell carcinomas. It would be difficult to convey this impression in a convincing manner, even with the help of numerous illustrations, and we will not endeavor to do so in this preliminary report. However, in an attempt to summarize the succession of events observed in the treated skin, the following could be stated: After the phase of acute or sub-acute fowl pox, continued treatment by methylcholanthrene resulted in a further hyperplasia of both epithelium and chorion. The former, in which vanishing inclusion bodies sometimes still could be discerned, was highly disorganized and had a blurred appearance, also characteristic of the acute pox lesions after hematoxylin-eosin staining (FIGURE 4). This easily identified epithelium seemed to evolve in two ways: first, invading the chorion in a diffuse manner and; second, integrating the base of the early papillomas that soon developed.

In both cases in later phases, this epithelium seemed to organize itself so that its cells could be discerned in a neat manner. Early keratinization was observed and the lesions then could give an impression of early malignancy. After this phase, images more and more nearly approaching those of squamous cell carcinomas were evident, until unmistakable cancers



FIGURES 12 AND 13. Microscopic images of squamous cell carcinomas that developed in the chickens treated with methylcholanthrene. Magnification: 125X and 400X.

finally developed. Some, or even all of the phases described above often could be seen in the same lesion.

A comparable sequence of events could be observed in the case of angiomas. The earliest images here were nests of highly distended capillaries which appeared as clefts in the hypertrophic and richly vascularized chorion from the fowl pox lesions. Progressively larger lesions evolved after this phase and ended in large typical angiomas.

On the Recovery of Free Fowl Pox Virus from the Dermal Lesions Developing after Treatment with Methylcholanthrene. Virus was recovered from all lesions that developed after treatment with the carcinogen. The lesions were florid and regressing pox, papillomas, angiomas, and squamous cell

TABLE 2
RECOVERY OF FOWL POX VIRUS FROM SKIN LESIONS OF CHICKENS TREATED
WITH METHYLCHOLANTHRENE
(RESULTS IN BIRDS THAT LIVED LONGER THAN THREE MONTHS)

<i>Experiment</i>	<i>No. of chickens</i>	<i>No. of methylcholanthrene paintings</i>	<i>Virus recovered in</i>	<i>No. of biopsies</i>	<i>Virus recovered in</i>
51 B	18	137 in 14 months* (average)	13 (72.0%)	65	20 (30.8%)
51 C	16	155 in 13 months* (average)	12 (75.0%)	81	23 (28.4%)
51 E(a) 1	20	131 in 14 months* (average)	10 (50.0%)	160	14 (8.8%)
51 E(a) 2	20	123 in 14 months* (average)	11 (55.0%)	145	14 (9.7%)
51 E(b) 1	20	82 in 9 months	13 (65.0%)	105	15 (14.3%)
51 E(b) 2	20	82 in 9 months	10 (50%)	87	11 (12.5%)
Total	114		69 (60.5%)	643	97 (15.1%)

* 82 of the paintings given in 3¼ months.

carcinomas. The presence of free virus in these lesions, tested on chicks in the manner described above, manifested itself by the development within a week (often in three or four days) of typical, florid pox, especially around the feather follicles (FIGURES 2 and 3). Further passages increased the activity of the virus so that clean-cut lesions were always evident in two or three days, attained their maximum size a few days later, and had regressed by the 20th day.

The results summarizing the recovery of virus are given in TABLE 2, which includes birds that lived longer than three months after treatment was started. It is seen that virus was recovered in 50 per cent to 75 per cent of the chickens in the different experiments. The following considerations, however, are pertinent for a more exact estimate of the presence of free virus in the treated birds:

(1) The presence of free virus in skin lesions was not investigated sys-

tematically at regular intervals in all birds, and the data showed that the demonstration of virus in each bird generally was in direct relation with the attempts to show its presence in the biopsied tissue. In experiments 51 B and 51 C, therefore, virus was recovered in 16 out of 17 chickens from which four to ten biopsies were taken, although it was recovered in only nine out of 17 chickens from which one to three biopsies were taken.

(2) In a number of chickens from the skin of which virus was not recovered, lesions grossly suspicious of pox were observed early in the experiment, and histological study disclosed in some of them characteristic images of pox infection. Further, comparable revealing images were observed, in the same or in other chickens, in the lesions that developed later in the treatment.

(3) In still other cases in which virus was not demonstrated in the skin, it was recovered from viscera at death.

(4) Frequently, lesions developed in only one of the three chicks on which the extracts from biopsied tissue were tested, and it is logical to suppose that virus would have been revealed more frequently had more chicks been used for the tests.

From the above, it seems extremely likely, if not absolutely certain, that fowl pox virus was activated by methylcholanthrene in every one of the chickens treated for long periods of time.

Another problem to be considered is the frequency with which virus was recovered from lesions in the same chicken. On the one hand, data in TABLE 2 indicate that virus was recovered more often in experiments 51 B and C than in experiment 51 E. On the other hand, analysis of individual cases showed that, in both groups, virus was sometimes recovered as often as three or four times in seven or eight biopsies taken from the same bird in periods 3 to 15 months. Finally, the same number of biopsies sometimes failed to demonstrate virus in other birds of the same groups. Judging by the available data, it would seem that three factors exist which must be subjected to careful scrutiny, since they may influence the results. They are the intensity and the length of treatment by the carcinogen; the time at which virus is again investigated following a previous virus recovery; and the type of lesion in which virus is investigated.

The problems above are of interest for the following reasons: If, in some circumstances, free virus was frequently recovered during long periods from lesions of the same host, it seems logical to assume a defective immunological response of the host to the virus. On the other hand, if, apparently in the same circumstances, virus was not recovered from lesions, one could suspect that it was present in a masked state.

Presence of Free Virus in the Skin and Viscera Early in the Treatment, and In Viscera at Death. In 50 chickens of experiments 51 C and E (not included in TABLE 2) that died or were killed two to three months after treatment was started, virus was recovered in the skin of nine of the birds. In four of them, the only discernible lesion was a slight thickening, after the birds had received 25 to 70 paintings.

Virus was investigated in the liver, spleen, and kidney of 33 chickens that

died from the 8th to the 20th months of the experiment, and was recovered in nine cases. In ten of the birds, virus had not been recovered from skin during life or at death.* Virus was also investigated in the viscera of 40 chickens of experiment 51 E that were killed, and was recovered in two cases after the birds had received 63 paintings in about three months. The liver and kidneys from which virus was recovered often showed extensive degenerative lesions, but it is impossible to say whether they were due to the carcinogen, the virus, or both agents.

In conclusion, free virus was present in the skin and viscera of at least some of the chickens since the early stages of treatment, and was also present in the viscera of approximately a third of the birds that died many months later.†

Presence of Free Virus in the Untreated Control Chickens and Chickens Injected with Testosterone. Infection by fowl pox virus, as manifested by the development of gross lesions, was observed in only one of the 73 untreated control chickens. This particular bird, from experiment 51 E, developed a typical, florid pustule, from which virus was recovered, in the skin of the right breast at the eighth month of the experiment.

In the same experiment, 51 E, the presence of virus was investigated in a total of 52 samples of grossly and microscopically normal skin, secured by biopsies from the 42 untreated controls at periods ranging from 3 to 10 months after the experiment was started. Virus was recovered in two cases. Also, in 11 of these control chickens that died, virus was recovered from the viscera in two birds, at the third and eighth month.‡

Of the control chickens injected with testosterone, nine of 27 in experiment 51 B, and one of 17 in experiment 51 C developed gross suspicious lesions which were observed at the same time and in the same location, and had the same appearance as the lesions in the test birds.

In summary, fowl pox virus was found to be present in a small minority of the untreated control chickens and probably in a higher proportion of the birds treated with testosterone. The presence of virus in these cases is not surprising if one considers that the control chickens lived in close contact with the infected treated birds, and were subjected to repeated injury by plucking of their feathers.

Ineffectiveness of Isolation in Preventing the Development of Fowl Pox in the Treated Chickens. Half of the chickens of experiment 51 E [groups (a)

* We may point out that if these cases are included in the figures of TABLE 2, the incidence of virus recovery in chickens would be raised to 83.5 per cent in experiment 51 B, and to 70 per cent and 55 per cent in experiments 51 E (b) 1 and (b) 2, respectively.

† The recovery of virus, early in the treatment, from skin and viscera poses the problem of whether the fowl pox infection was activated simultaneously in the skin and viscera, or whether it was first activated in one tissue, with subsequent localization in the other. The problem is of interest, first because its solution may indicate the tissue in which the latent pox virus is located; and second, because, if the virus was first activated in the viscera, it would indicate that one of the first local effects of the carcinogen, possibly the first effect, would be the localizing, in the tissue where it is applied, of a virus activated elsewhere by the carcinogen itself.

In this respect, we may well cite unpublished results from our laboratory which showed that vaccine virus, injected intravenously into rabbits, localized itself preferably in the areas of the skin which had been painted with methylcholanthrene. The experiment is far more striking when carried out in chickens a few weeks old. In these instances, the virus localized itself strictly and conspicuously in the skin treated with the carcinogen. No localization occurred in untreated control chickens of the same age similarly injected with the virus.

‡ We may add to the above that the presence of virus was also investigated with negative results in the skin of 15 adult chickens recently purchased from the same dealer supplying many of our birds.

1 and (b) 1] were hatched and raised in conditions of isolation.* Groups (a) 2 and (b) 2 were kept in a surrounding contaminated by the presence of other birds purposely infected with fowl pox. The birds kept in isolation were given 82 paintings either in $3\frac{1}{4}$ months [group (a) 1] or nine months [group (b) 1], and the same procedure was followed with the birds of groups (a) 1 and (b) 2. Results from passages of biopsied tissue, carried out in the usual manner, soon showed that free virus was equally present in the chickens kept in isolation and those kept in a contaminated surrounding.

Experiments on Adult Chickens. The experiments described above were repeated on 28 chickens first treated with the carcinogen when ten months old. Although results have not been subjected to final analysis, one can advance the observation that the results were essentially the same as those when chicks were employed at the start of the experiments. Virus was recovered in 19 of the birds. The number of skin samples secured by biopsies was 139. Virus was recovered in 39 of them. Papillomas, angiomas, and squamous cell carcinomas developed at the ratios of approximately 90 per cent, 10 per cent and 20 per cent.

Experiments on Pigeons. Fundamentally, what was observed in chickens was found to hold true in the experiments carried out so far in pigeons. Forty-five of these birds were given 15 to 63 methylcholanthrene paintings twice a week in periods ranging from three to eight months. Pigeon pox virus was recovered from 28 of the birds. Papillomas and cancers, but not angiomas, developed in 100 per cent and in 15.6 per cent, respectively.

Two of the pigeons that died with cancers seven months after the experiment was started showed widespread metastases in liver and lungs.

Discussion

If a virus latent in a host is regularly activated by a carcinogen, and if the same virus, under certain circumstances, is later recovered in the neoplastic lesions that the same carcinogen has caused to develop, it may perhaps be justifiable to suspect that the virus has been instrumental in some way in the genesis of some or all of the lesions. The suspicion may increase after considering that the virus is endowed with a strong, although ephemeral, power to stimulate cell growth,† and that, apparently at least, there is a gradual transition from the early acute lesions currently induced by the virus to the late neoplastic lesions.

The above facts are fundamentally those which have been observed in our experiments in which the skin of chickens and pigeons was repeatedly treated with methylcholanthrene.

It is obvious, however, that the facts could be interpreted in a far more conservative way by assuming that the carcinogen has activated the virus and induced neoplasia by two entirely unrelated mechanisms, and that, if

* The isolation rooms were sterilized by means of antiseptic solution and ultra-violet light. Access to the rooms was gained through anterooms where the attendants changed coats and put on disinfected gloves and boots. The eggs, secured at the 17th day of incubation, were fumigated with an antiseptic solution and hatched in the isolation rooms in previously sterilized incubators.

† The fact that fowl pox is also known by the term *epithelioma contagiosum* is quite revealing in this respect.

virus is found in the lesions, it is by no means a causative agent, but a rider that infects the neoplastic tissue and changes its morphological features somewhat.

A crucial experiment to solve the problem would be one proving that, in hosts not carrying the virus, methylcholanthrene is ineffective in inducing all or some of the lesions that developed in our infected chickens.* In the text, we have described an attempt to secure such hosts by hatching and breeding chicks in isolation. The experiment failed either because the virus was present in the egg from the start or because, despite precautions, contamination from without took place. Other experiments of the same sort, carried out in more extreme conditions of isolation, are now in progress.

A new approach to the problem, based on recent findings from our laboratory, may exist, however. Basically, these findings concern: (a) defective development by chickens of resistance to re-infection by fowl pox virus, a fact which could be surmised by the frequent recovery of virus from the same chicken during long periods; (b) characteristic morphological features of the lesions, induced by intentional re-infection of the treated chickens, resembling some of the lesions that develop at random during treatment with the carcinogen; and (c) the state of the virus, free or masked, in these lesions compared to the state of the virus in the lesions of primary infection.

Further work will probably decide whether fowl pox virus is involved in the genesis of the neoplastic lesions. If it is not involved, the point of main interest to those in the cancer field, of the findings reported here, would be that the same process leading to carcinogenesis also leads to activation of latent virus infection, and this revived virus, thriving in the neoplastic tissue, changes its morphological features as it conceivably could also change its biological characteristics. In the virus field, the problems posed concern: (a) the nature of the activating effect of the carcinogen on the virus; and (b) the long persistence of the virus, in a free or masked state in chickens treated with carcinogens, in relation to the immune reactions of the host.

If it appears that fowl pox virus really was instrumental in the development of some or all of the neoplastic lesions described, the thesis would have been proved that an ordinary virus, activated by a carcinogen and infecting a tissue prepared by the same carcinogen, can cause cancer. The field then would be open to investigate other ordinary viruses as possible causes of neoplasia; and to look for such viruses, not as riders but as causative agents, in tumors and other tissues of the cancerous host.

Summary

(1.) In practically every case, treatment of chicks, adult chickens, and pigeons with methylcholanthrene activated a latent fowl pox infection which was first manifested by the appearance, strictly in the painted dermal area, of typical acute lesions from which the pox virus could be recovered by passage. Virus was also recovered, after death, in the viscera of the birds.

* Such an experiment may have been carried out. In England, Peacock and Peacock¹⁰ were unable to induce cancers or other lesions in a large number of chickens, despite long treatment with a variety of chemical carcinogens. In one of the experiments, 20 brown Leghorn pullets, three months old, were painted 84 times at weekly intervals with a one per cent solution of 9:10-dimethyl-1:2-benzanthracene in either acetone or benzol on the undersurfaces of the right and left wings.

(2.) On continuation of the paintings, papillomas, angiomas, and squamous cell carcinomas developed in the treated skin. The cancers that developed early proved to be of low malignancy, but those that appeared many months after starting the treatment induced widespread metastases and could be transplanted to other hosts.

(3.) Apparently, at least, there was a gradual microscopic transition from the stimulated epithelium and the very vascularized chorion of the acute pox lesions to squamous cell carcinomas and angiomas, respectively.

(4.) Fowl pox virus was recovered often from the neoplastic lesions, but the frequency in detecting the virus from successively-appearing lesions in the same host seems to be conditioned by a variety of factors.

Whether the activation of fowl pox virus is a phenomenon directly involved in the process of carcinogenesis, or whether it is an event concomitant to carcinogenesis which may affect, however, the morphology, and conceivably the biology, of cancers and other lesions, was discussed.

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STUDIES ON FOWL LYMPHOMATOSIS

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Introduction

The disease complex, fowl lymphomatosis, is of particular interest to investigators concerned with the etiology of cancer because at least one form of this disease is truly cancerous. It is caused by a virus-like agent and, furthermore, is contagious in a manner similar to that of other contagious diseases.

Three forms of lymphomatosis have been recognized. The form which shows lymphoid deposits in any of the nerve trunks is known as neural lymphomatosis. When this condition becomes sufficiently extensive, a loss of function results. Ocular lymphomatosis is the term applied to the form of the disease in which there are similar lymphoid deposits in the eye. The iris is usually involved, becoming constricted, immobile, and gray in color with resulting blindness. When any of the viscera show extensive lymphoid involvement, the form of the disease is known as visceral lymphomatosis. Among others, terms which, have been applied to this latter condition are lymphocytoma, lymphosarcoma and lymphoblastoma.

Osteopetrosis gallinarum often occurs in birds having visceral lymphomatosis. It is also present as a transmitting entity in some propagated strains of visceral lymphomatosis. Because of this association some investigators have classified this form of the disease as osteopetrotic lymphomatosis. However, the principal lesion is not lymphocytic but is an excessive formation of hard bone caused by a hypertrophic activity of the periosteum and endosteum.

These conditions may occur separately, although they often occur in combination. The more usual naturally-occurring combinations are neural-ocular, neural-visceral, and visceral-osteopetrotic. Combinations of three and four conditions have also been found. Some investigators have claimed that these four entities have a common etiology. There is much evidence, however, especially in the field of epornithology and also that obtained in the study of the transmission characteristics, which indicates that the etiology of the four pathologic entities may in fact be distinct. Visceral and neural lymphomatosis are the two most important diseases of this group. They occur throughout the world and it has been estimated that they cause an annual loss to the poultry industry of over 75 million dollars in the United States alone. It is estimated that the visceral form is responsible for at least two-thirds of this loss.

This discussion will be confined primarily to visceral lymphomatosis although the nerve and osteopetrotic forms will also be considered. Whether visceral lymphomatosis may be classified as a malignant neoplastic disease has been questioned by some investigators. Time does not permit the presentation of evidence relating to such a classification. It may be stated, however, that the majority of evidence suggests that a bird which develops a

tumorous condition of the viscera, made up primarily of immature cells of the lymphoid series, will die in a comparatively short time. The question of whether this tumor is a true neoplasm has been amply reviewed by DeOme,¹ who concluded that the four properties of neoplastic growth, namely: (1) uncontrolled and unorganized growth; (2) metastasis; (3) invading, displacing, or replacing surrounding normal tissue; and (4) a predilection for certain tissues and locations; are all characteristic of avian lymphomatosis. Most lesions seen in cases of ocular and neural lymphomatosis, however, have characteristics which are more typical of reactions to foreign infectious agents than of true neoplasms.

Pre-tumorous Host Reactions

In studies of the histopathogenesis of this disease, many investigators have questioned the significance of the many lymphoid areas found in various organs in relation to lymphomatosis. Most workers in this field considered them to be normal, with functions similar to the lymph nodes of mammals which, as such, are absent in the chicken.

Recent extensive work by Lucas and co-workers²⁻⁷ and by Oakberg *et al.*^{8, 9} indicates that all such lymphoid areas should be classified as abnormal, because many of them (a) show invasiveness, (b) destroy tissue, (c) plug vessels, or (d) destroy nerve tissue supplying an organ. These lymphoid areas are found in almost all chickens of all age groups from baby chicks to adults. The areas increase in size and number with age and are found in many other species of domestic and wild birds, many of which have not shown gross manifestation of lymphomatosis. Nevertheless, a significant correlation was obtained in the analysis of data relating to incidence of lymphomatosis among different strains of chickens and the amount of lymphoid tissue in the pancreas of 600-day-old survivors. Furthermore, chickens inoculated with the filterable agent of visceral lymphomatosis had five times as much lymphoid tissue in the pancreas at 100 days of age as the non-inoculated controls.¹⁰ Is it possible that these ectopic lymphoid foci found in various organs in varying amounts are the result of a reaction to a systemic infection with the agent of lymphomatosis, and the later neoplasia has its beginning in one of these foci?

Investigations so far have suggested certain possibilities and probabilities although it may be questioned whether the agent of lymphomatosis is the only pathogen to cause initiation and/or activation of these areas in the body.

Modes of Natural Transmission

Many difficulties are encountered in studies on the transmission of lymphomatosis. Among them are: (1) the long incubation period of the disease; (2) lack of experimental stock, free from infection, and (3) the presence of various forms which may or may not be related etiologically.

Through the Egg. The fertile egg has long been incriminated as a natural means of transmitting lymphomatosis. Numerous investigators have reported indirect evidence supporting the claim that this disease is egg-borne.

Recently completed experiments by Cottral and co-workers¹¹ and Cottral^{12, 13} show conclusively that the causative agent of visceral lymphomatosis is present in the chick embryo and embryonic fluids. The presence or absence of the agent in embryonic material was determined by inoculation of groups of 15 to 30 baby chicks of a line known to be highly susceptible to this disease although relatively free from infection when hatched, as indicated by the low incidence of the disease when the birds were raised in isolation.

Materials used for infectivity tests included liver suspensions of 15-day embryos, 18-day embryos, one-day-old chicks, liver filtrate of 18-day embryos, and amnionic fluid. Blood and tracheal washings of certain adult chickens which produced the embryos tested for infectivity were also used. The dams providing the source of embryonic material were chosen either at random or on the basis of a high or low incidence of lymphomatosis in their sibs and progeny. In either case, they showed no clinical evidence of lymphomatosis when the fertile eggs were laid to provide the embryos for testing.

Suspensions prepared from a pool of the livers of 15-day-old embryos, randomly selected from a highly infected population, produced a significantly* high incidence of visceral lymphomatosis in two of three experimental lots. When tests were made, using embryo livers of 12 individual dams which were selected because of a high incidence of lymphomatosis in their sibs, transmission was obtained in four of 12 tests. In a later experiment, four of the original 12 dams were retested. Embryo liver of two of the dams gave confirmatory results, whereas the other two reversed themselves; one became positive and the other became negative. Whether these differences are due to errors in measurement or to actual differences in embryos from the same hen has not been determined. Similar tests of embryo livers of dams maintained in isolation but still showing some lymphomatosis infection, resulted in two of nine tests giving significant transmission. Retests on five individuals gave confirmatory results. Seitz-filtered embryo liver material has given significant transmission in two of six tests. Preparations of liver from 18-day embryos and day-old chicks, and amnionic fluid, as well as the blood of some of the dams, have given positive results.

These data show conclusively that the filterable agent causing visceral lymphomatosis is present in the embryos of certain dams. They also show that it is present in the extra-embryonic fluid, in the circulating blood of some dams, and in day-old chicks, thus demonstrating that the agent is transmitted from parent to offspring through the egg.

The second significant result of the egg transmission experiments was the production of tumors with material arising from apparently normal individuals. All embryos used were grossly normal and all dams were clinically normal when the eggs were laid and they remained so for variable periods. During a period of approximately one year after the use of the embryos, only three cases of visceral lymphomatosis developed in the 39 dams. One case developed 38 days after the use of its eggs in the experiment, and the other two developed at 136 and 144 days. Furthermore,

* Based on a difference in the incidence of visceral lymphomatosis between the experimental and control lots with significance at the 5.0 per cent level or less, using chi-square test.

there was no apparent relation between death of dams from lymphomatosis and infectivity of embryonic material.

It is clear, therefore, that the agent of visceral lymphomatosis remains inapparent or latent in many grossly normal egg-laying chickens and at the present time, such infection can be demonstrated only by inoculation of suitable material into highly susceptible, yet relatively disease-free stock.

The practical significance of egg transmission, *i.e.*, the portion of the naturally-occurring disease which may be directly or indirectly attributable to egg transmission, remains to be determined.

Environment. Environmental factors also appear to play an important part in the transmission of lymphomatosis. The natural body excretions have been strongly implicated as a source of direct and indirect transmission.

Experiments by Water and Bywater¹⁴ showed that when chicks from lymphomatosis-free but susceptible parents were raised in isolation, there was no lymphomatosis or a low incidence, but when siblings were hatched and raised in direct contact with stock that had a high parental infection, the incidence of lymphomatosis was high. This high tumor rate was reduced by postponing contact until after the hatched chicks were removed from the incubator. It was further reduced by progressively increasing the isolation period to 30 days. Birds maintained in isolation for only 30 days had no higher rate of tumor development than those in isolation for the whole experimental period.

Proximity to infected old stock as a factor in natural transmission was emphasized by experiments of Hutt *et al.*¹⁵ They found that when alternate hatchlings from the same parental stock were brooded for the first two weeks at two different distances from adult stock, there was a consistent difference in mortality from lymphomatosis. Although all other genetic and environmental factors were apparently the same for the two groups, those brooded at the greatest distance from adult stock nevertheless had the least amount of lymphomatosis.

Cole and Hutt¹⁶ also showed that when chicks were raised in an area isolated from direct or indirect contact with other chickens, the mortality from lymphomatosis was reduced to a very low figure. This was a confirmation of earlier work by Waters,¹⁷ which showed that raising families of chicks in pen isolation greatly reduced the incidence of lymphomatosis.

Further insight into the contagiousness of this tumor has been obtained by experiments with lymphoid tumor strains derived directly from cases of naturally occurring visceral lymphomatosis.

On the basis of experience with the Rous tumor virus and the virus of erythrogranuloblastosis, titration groups were inoculated and maintained together in the same pen. Two titrations were conducted at about the same time with dilutions of 1:1 to 1:100,000. All inoculated groups developed a high incidence of tumor. The range was from 62 to 77 per cent with no relation to the dilution. Two groups of contact controls also developed a relatively high incidence of tumors, the percentages being 53.0 and 58.1. Only 5.9 per cent of the controls, maintained in a pen isolated from inoculated chickens, developed tumors during the same experimental period.

These results definitely indicate that much cross-infection between dif-

ferent inoculation groups and between inoculated and control birds must have taken place. Because of these results, an experiment to determine the effect of the length of the contact period on the extent of transmission was conducted. The results are presented in TABLE 1, which shows that 48 per cent of the 129 inoculated birds developed lymphoid tumors of the viscera; whereas 70 per cent of those in contact during the entire experimental period of 250 days developed tumors. Chicks that were kept isolated in a cubicle for the first 30 days, then placed with inoculated birds, developed a tumor incidence of 57 per cent. When this period was extended to 60 days, a tumor incidence of 21 per cent was obtained. Since only one of 51 isolated control birds died with a tumor, it may be concluded that much contact transmission took place and that the amount of this transmission was reduced when birds were maintained in isolated quarters while they

TABLE 1
THE EFFECT OF CUBICLE ISOLATION ON THE TRANSMISSION OF VISCERAL
LYMPHOMATOSIS FROM INOCULATED TO NON-INOCULATED SIBLINGS
(EXPERIMENTAL PERIOD 250 DAYS)

<i>Treatment</i>	<i>Number of birds</i>	<i>Per cent visceral tumors</i>	<i>Per cent osteo- petrosis</i>	<i>Average survival (days)</i>
Inoculated.....	129	48	31	104
Contact with inoculated group the whole period.....	57	70	0	180
Cubicle-isolated 30 days, then contact.....	30	57	0	205
Cubicle-isolated 60 days, then contact.....	29	21	0	189
Isolated pen whole period.....	51	2	0	155

were young. Isolation to 60 days of age did not appear to eliminate all contact transmission. Whether this residue was the result of inadequate isolation measures or too short an isolation period was not determined. In a second experiment, however, in which the group was isolated for 90 days and ultraviolet lamps with high germicidal emissions were used, there was no evidence of any contact transmission.

Experiments with the naturally-occurring disease and the induced tumor leave no doubt that this is a contagious tumor capable of being spread from chicken to chicken. The means and avenues of spread need much more investigation. Early experiments have implicated the feces. Recent experiments¹⁸ have shown that tracheal and nasal washings of lymphomatotic birds induced tumors in susceptible chickens, thus suggesting that the respiratory route may be important in the spread of this disease.

The Nature of the Agent

Transmission with Cellular and Cell-free Preparations. Although visceral lymphomatosis is the most common neoplasm of birds and although it appears to be a contagious disease, many investigators over a long period were unable to transmit it by artificial means. In the last few years, however, it has been amply demonstrated that it can be propagated from most

naturally-occurring cases by use of cellular suspensions.¹⁹⁻²² It has also been conclusively shown that some of these tumors contain a filterable agent which will reproduce the tumor²³⁻²⁵ when injected into a suitable host. Why some tumors apparently do not contain a transmitting filtrable agent, while others, similar in appearance, do, has not been determined. It is probable, however, that the agent is present in all tumors of visceral lymphomatosis, although it is present in an inactive form in some. This is indicated by results with a tumor strain which gave no transmission with filtrates in the first transplant passage; although cell-free preparations gave a high rate of transmission in the fifth passage, an apparent loss in filterability may also occur. Two lymphoid tumors have shown a highly active filterable agent at the first few transplants, but an inactive or only slightly active agent at later transfers. These variations may be more apparent than real, since little is known concerning the variation in potency of the agent in the tumor during its growth and development. It is also quite possible that some lymphoid tumors are not caused by the agent of visceral lymphomatosis and therefore could not be expected to contain this agent.

In certain respects, this tumor is similar to the more widely known Rous sarcoma, although many important differences exist. Tumors of visceral lymphomatosis, after several cellular transfers, will consistently produce a tumor at the site of injection or implantation and produce extensive tumorous involvement of many of the visceral organs by metastasis or cell translocation. The tumor at the site of injection may become palpable as soon as four days after injection, and death, due to visceral involvement, may occur as early as six days after inoculation. Tumors grow in all or nearly all birds implanted, although the incidence of death as a result of metastasis depends largely on the innate resistance of the host. Filtrates of the same tumor give a different result. Irrespective of the route used, tumors do not develop at the point of inoculation, but one or more of the visceral organs becomes tumorous after a long incubation period. The involvement may be diffuse, focal, or a combination of the two types. When the involvement is diffuse, the bird may die within a few days after gross changes start, but when focal tumors develop, the bird may survive for weeks before death eventually results. Birds inoculated during the first post-hatching week start dying with typical tumors at about two months of age. The death rate gradually increases so that it is about one per cent per day by four months of age. The incidence of tumors in susceptible inoculated stock at 200 days will vary between 50 and 95 per cent.

The age at inoculation has a marked effect on the tumor incidence. In one experiment with Strain RPL 18, the results of which are presented graphically in FIGURE 1, five age groups, varying from two to 114 days, were inoculated with the filterable agent of a common source and at the same time in doses directly proportional to their size. The data show that the tumor incidence declined with an increase in age from a high of 95 per cent for the two-day age group to a low of 31 per cent for the 114-day group. The average survival period of those that developed tumors showed no particular relation to age at inoculation, however. These data indicate, therefore,

that it takes about as long for grown chickens to develop tumors as it does for baby chicks, but the incidence is much less.

Heritable factors also greatly influence the tumor rate. Certain lines or strains of chicks appear to be much more susceptible than others. This is especially true of inbred White Leghorns developed by Waters.²⁶ By intensive inbreeding over a period of nine years he has obtained susceptible and resistant lines of chickens. For the 1949 population, the most resistant line had an incidence of 11.1 per cent, and the most susceptible line had lymphomatosis in 40.9 per cent of the birds.

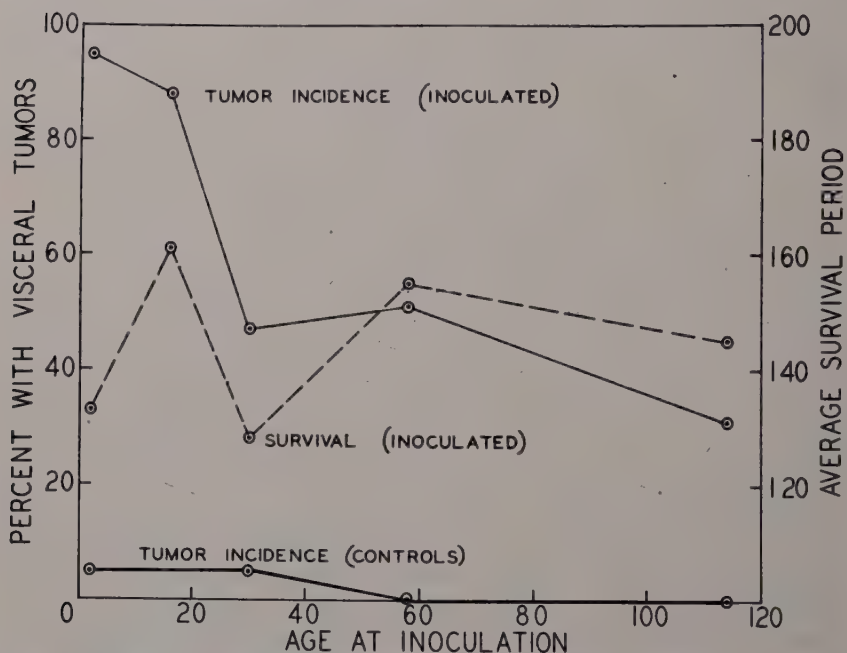


FIG. 1 EFFECT OF AGE AT INOCULATION ON TUMOR INCIDENCE AND SURVIVAL PERIOD.

FIGURE 1

Only a limited amount of data has been obtained on the viability of the tumor agent, as well as on other biological and physical properties. Preliminary data on viability have been obtained with the use of crude cell-free preparations of tumorous liver, using phosphate buffer as the diluent. In one test, the tumor incidence produced by fresh undiluted inoculum was 78 per cent and, for inoculum diluted 1:100, the incidence was 38 per cent. After the inoculum was frozen and stored at -76°C for 405 days, the incidence for the undiluted material was 79 per cent and, for the 1:100 diluted material, 63 per cent. In a second test, frozen inoculum was stored at -76°C for 50, 125, and 141 days and, upon inoculation, produced tumor incidences of 80, 80, and 82 per cent respectively. The results of both experiments indicate that there was little, if any, loss of activity of crude

preparations during storage in a CO₂ icebox. In fact, there is some indication, in the first test, of an increase in potency upon freezing and storage.

Lyophilization of crude preparation appears to result in some loss in activity. In one test, crude phosphate buffer tumor extracts were frozen and stored at -76°C for 403 days. Other tubes of the same preparation were lyophilized and stored at 2°C for the same period. The frozen inoculum produced tumor in 79 per cent of chickens inoculated and, when diluted 1:100, it produced tumors in 63 per cent. On the other hand, the lyophilized material, when reconstituted and injected into similar test birds, resulted in an incidence of 64 per cent when used undiluted and only 20 per cent when diluted 1:100. The latter result is not significantly different from that for the controls, thus indicating that much loss of activity resulted in the lyophilized samples.

Survival of the tumor-producing agent when subjected to other treatments has also received some attention. The agent in crude preparations, when heated for 30 minutes to a temperature of 55, 60, 65, or 70°C, was completely inactivated. Lyophilized material heated to a temperature of 50, 65, 80, or 95°C was also completely inactivated. The agent is also sensitive to formaldehyde and to ultra-violet irradiation but appears to be little affected by as much as 12,050 r units of X rays.

Whether the visceral tumor agent may be classified as a typical virus has been questioned by some investigators. Such a classification would depend to some extent upon one's definition of the term "virus." There is no question that the visceral tumor agent has many of the biological and physical characteristics usually ascribed to viruses, although many of its properties remain to be investigated. Although filtration experiments have shown that the agent will readily pass bacteria-retaining filters and that it may be sedimented by high-speed centrifugation, its size and shape have not yet been determined. Its antigenic characteristics are still in question. Whether antibodies occur naturally or may be induced by injection has not been fully explored. The few experiments conducted on this phase have been uniformly negative.

Variations. This discourse would be grossly incomplete without some reference to the variations in manifestations that have been obtained.

When tumors of visceral lymphomatosis are propagated with cellular suspensions, the strain remains essentially static since this type of transmission is only a cell transplant which allows the continuous reproduction of the same cells. When passages were made with filtrates prepared from tumors of early developing cases, however, some of the birds died suddenly at less than 100 days of age with alterations suggestive of leukemia. In such cases, large round neoplastic cells are located almost entirely within the vascular bed. Their chief location is in the liver sinusoids, and blood smears show large numbers of the same cells in the peripheral circulation a few days before death. This type of involvement is in contrast to the tumorous condition typically found in visceral lymphomatosis and in most passages of strains derived from it. In this condition, the lymphoid accumulations may be located diffusely throughout the organ or may be confined to focal areas. In either case, the normal tissue is replaced, the

organ greatly enlarged and the neoplastic cells located almost entirely extra-vascularly. Only occasionally are the tumor cells found in the peripheral circulation and then only in acute cases.

If this intravascular variation is a true leukemia involving the primitive blood cell, the causative agent must be similar to, or identical with, the well-recognized virus of erythrogranuloblastosis,^{27, 28} since the same primitive cell (hemocytoblast) appears in varying proportions in the circulation in cases of the latter disease.

Under natural conditions, erythrogranuloblastosis is a rare disease, in comparison with lymphomatosis. This form of leukemia, like sarcoma, but in contrast with lymphomatosis, does not appear to be contagious. The only recorded observation in disagreement with this general statement is that of Hamilton and Sawyer,²⁹ who reported an outbreak of erythroblastosis which reached epizootic proportions. It seems significant, however, that many of the present strains of erythrogranuloblastosis arose in the first few or several passages of a strain derived from a naturally occurring case of lymphomatosis. Good examples of this are the present Beltsville strain "A" erythrogranuloblastosis which Dr. Beard and co-workers are using. It originated in the passages with material from two cases of neural lymphomatosis. A second example is Strain RPL 3. The leukemic form appeared in the sixth to eighth passages with blood originating in three cases of ocular and neural lymphomatosis.

Not only do we get the appearance of leukemia in passages and sub-passages of strains derived from cases of lymphomatosis but also of other but related tumors. Furth³⁰ and Burmester²⁴ have described the occurrence of endotheliomas, hemangiomas, and osteochondro-, fibro- or myxosarcomas. These types of tumors have usually occurred in passages of leukemic strains. There is some suggestion of sequential tendency in the occurrence of these variations. The leukemic forms occurring in passages of lymphomatosis and the special tumors arising in passages of the leukemic forms nearly always contain a filterable transmitting agent.

In seeking possible explanations for this variation one should include the following possibilities:

Due to an increased virulence or dosage of virus, an increased susceptibility of the host, or a combination of these factors, neoplasia results after a shorter incubation period, and since all inoculations are made during the first post-hatching week, neoplasia occurred while the birds were young. The reaction of young chickens to the virus may be an acute intravascular leukemic type, although the more chronic extravascular aleukemic type results in older birds. If this idea were extended to include all forms of lymphomatosis, leukemias, and related tumors, one would essentially be describing the hypothesis first presented by Ellermann,³¹ which ascribes all forms to a single multipotent agent.

A second possibility is that the virus of visceral lymphomatosis has a strong tendency to mutate toward a leukemia-producing virus, which occurs and is manifested during rapid serial passage, and the latter may mutate further to produce other types of neoplasia. Furth,³⁰ Oberling and Guerin,³² Duran-Reynals,³³⁻³⁵ and Shrigley³⁶ have obtained modifica-

tions. Some have occurred by the use of deliberate procedures and others arose spontaneously. These results were usually interpreted as changes in the original virus.

A third possibility is that the tumor strains contained other neoplastic agents from the beginning or acquired them during serial passage from the host or the laboratory. It has been shown that the agent of visceral lymphomatosis is carried in a latent state by many apparently normal chickens and, on the basis of experimental results, it was suggested²⁴ that chickens, although showing evidence of only one kind of tumor, may carry several oncogenic agents in a latent form.

On the basis of immunological evidence, Andrewes³⁷ thought that all strains of sarcoma were different and, because of variations in pathological manifestations, Furth³⁰ believed that all of his leukotic strains were different. The proposition then arises that each naturally-occurring case represents a related, although different virus, an idea connected with virus tumors for some time. Such a possibility, however, does not seem logical since visceral lymphomatosis is a contagious disease which spreads from bird to bird in the same pen and from parent to offspring, *i.e.*, it is a horizontal and a vertical infection. In this process, the same agent or agents become common among the population, producing predominantly one or another or a combination of two or more disease entities, depending possibly on other factors, such as the genetic or environmental.

Much has been written and many experiments have been performed in an attempt to elucidate the basis for variations obtained with oncogenic agents. Conclusive results supporting an adequate explanation have not yet been presented. Furthermore, critical experimental approaches for one reason or another thus far have not been conducted. The major handicap possibly is the lack of experimental stock which is known to be free of all oncogenic agents.

Summary

Visceral lymphomatosis, probably the most important disease in chickens, is a contagious malignant neoplastic disease caused by an agent which has many of the properties of the common viruses. This agent is spread and produces the disease by direct contact and probably by the aerogenous route. It is also spread from parent to offspring through the embryonated egg and is found in a latent state in many apparently normal adult chickens.

Visceral lymphomatosis may be propagated with tumor cell suspensions or with filtered tumor extracts. Known properties of the agent would place it in the virus category of disease agents. It is a rare type, however, since it produces malignant tumors and is also highly contagious.

In the serial passage of lymphomatosis with the infective agent, variations in pathological manifestations have occurred which appear to be directional. The new forms may be reproduced by filtrates, indicating a change in some property of the agent, or the expression of a different agent which had been in a latent state.

Progress in the study of this and other phases of lymphomatosis is greatly impaired by the lack of experimental stock free of oncogenic agents.

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BIOLOGICAL STUDIES ON THE MAMMARY TUMOR INCITER IN MICE

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This paper concerns two unrelated series of experiments which were designed to investigate the propagation of the mouse mammary cancer virus and test wild house mice for the presence of the virus. In both studies, the natural route of transmission of the virus was used, *i.e.*, from generation to generation by way of mother's milk, since it is one of its most interesting biological characteristics.

The first investigation is discussed briefly because the procedures and results have already been published.¹ They are presented here as evidence that the propagation of this virus is not understood clearly and deserves further study.

New-born mice of the high mammary tumor strain C3H were kept with their mothers for 24 to 40 hours after birth and were then transferred to low mammary tumor strain C57 black foster mothers. When the fostered mice were two to three months of age they were bred to brothers and their female offspring divided into two groups. The first group, designated fostered mice, remained with their mothers for 24 to 40 hours after birth before being transferred to a C57 black foster mother. The second group, designated control mice, remained with their C3H mothers during the entire nursing period. This procedure was followed for five generations of brother to sister matings. The results of only one experiment are shown in TABLE 1 since it contained the largest number of animals and is typical of four others. It is seen that mammary tumors ceased to appear in the majority of mice after two or three generations.

These experiments were performed to ascertain whether, in common with other viruses, the mammary tumor virus increased in amount in each host. The findings suggested a dilution of the agent in successive generations of fostered mice. Various interpretations are possible, ranging from neutralization of the virus by C57 black milk to a negligible propagation of the virus.

Green, Mossey, and Bittner² have reported the results of a similar experiment in which a more direct approach was used. They infected mice by intraperitoneal injections of a mammary tumor filtrate and then attempted serial passages by administration of filtered extracts of lactating mammary glands. Fifteen mice were used in each of four passages. In the first passage, 11 developed tumors; in the second, six, and in the third and fourth, none.

Other investigators have reported interesting results which may have some bearing upon the propagation of the virus. Bittner³ suggested that the virus may be more concentrated in older mice and also reported⁴ an observation which implied a dilution of the virus in successive generations. Strong⁵ noted a decline in the incidence of mammary tumors in strain A

TABLE 1.
DISAPPEARANCE OR INACTIVATION OF THE MAMMARY TUMOR VIRUS IN A LINE OF FOSTERED STRAIN C3H BREEDING FEMALES*

Generation	5F + 11									
F	6C + 9	7C + 9	8C + 11	9F + 14	10F + 11	31C - 24	32C - 25	33C + 11	34F + 10	35F + 14
F ₁	50C + 13	43C + 20	41F - 25	42F - 25		40C + 11	39C + 12	38F + 16		
F ₂	60F - 21	61C - 23	62F - 21		63F - 20	68C - 21	60F - 20	70F - 20	58F - 8	59F + 8
F ₃	77C - 23		105F - 23		87C - 24		85F - 17	94F - 21	101F - 13	82F + 16
F ₄	78F - 19		106F - 21		88C - 18		86C - 23	93F - 25	102F - 24	83F - 24
	79F - 28		107C - 27		89C - 12			96C - 24	103C + 9	84C - 20
					90F - 25			97C - 16		
					91F - 24					
					92F - 23					
					93F - 25					
					125C - 14					
F ₅	111C - 23		121C - 23		126C - 16			132F - 25	120C - 25	115C + 21
	112F - 22		122F - 25		130C - 25			133F - 25		
	113F - 22		134C - 19		135C - 17					
	114F - 24		136C - 25		137F - 24					
			138F - 25		139F - 20					
			140F - 22							

* After the number of each mouse, F denotes mice foster-nursed after 24 to 40 hours with C3H mother, C denotes mice that remained with C3H mother for entire nursing period, + denotes the occurrence of mammary tumor, — denotes death without tumor. The age in months is given after the + or — symbol.

mice. Workers at the Jackson Memorial Laboratory^{6,7} found that the virus may be inactivated in blood during late pregnancy. Murray and Warner⁸ reported the disappearance of the virus from a female of the Marsh albino strain based upon the absence of mammary tumors in 351 of her descendants. It is interesting to note that Shabad,⁹ who does not look upon this agent as an exogenous virus but considers it a substance of endogenous origin, believes that inbreeding through many generations promotes the concentration of the substance in certain lines of mice. The propagation of the mouse mammary tumor virus obviously deserves further consideration.

The second investigation, reported here in more detail, was an effort to determine whether the wild house mouse harbors the virus. The necessity for this investigation became apparent when it was ascertained that certain inbred mice,¹⁰ or the hybrids of inbred mice,¹¹ show a high incidence of mammary tumors in the absence of the virus. The only pronounced difference between these tumors and those developing in mice carrying the virus is the later mean age at which tumors arise in animals that are free of the virus.

If wild mice do not possess the virus, then its presence in high breast cancer inbred strains could be considered a consequence of domestication, that is, an infection acquired in the laboratory. If the virus is carried by wild mice, then the establishment of inbred strains with varying degrees of susceptibility to the development of mammary tumors was dependent upon the selection of mice susceptible or resistant to infection by the virus. It would be of special interest if wild mice were to develop few mammary tumors and contain a virus of low activity, since this could be considered evidence that inbreeding led to an increase in the concentration or activity of the virus. This, in turn, would imply the importance of the genetic constitution of the host in efforts to reveal the presence of tumor viruses.

After several unsuccessful endeavors to breed wild house mice in the laboratory, it was learned that Dr. H. A. Schneider¹² has established a breeding colony at the Rockefeller Institute and he generously supplied sufficient animals to begin the study. By following the procedures outlined by Dr. Schneider, a number of wild mice were raised, and it was soon found that the females suckled newborn laboratory mice and *vice versa*.

A small number of wild mice were kept to ascertain their susceptibility to the development of spontaneous breast cancer. TABLE 2 summarizes the occurrence of tumors in 36 breeding and nine non-breeding females.* One mouse developed a mammary tumor which was not tested for the presence of the virus. This animal bore but one litter which consisted of three males. The frequency of pulmonary tumors in these wild mice was of some interest because this type of tumor was encountered frequently in laboratory mice¹³ before the establishment of inbred strains; and subsequent studies¹⁴ have revealed the importance of genetic influences in the spontaneous occurrence in the induction of this tumor. A review of the literature has failed thus far to reveal any information concerning the incidence of tumors in the wild house mouse.

* All tumors in this experiment were diagnosed histologically by Dr. Thelma B. Dunn of the National Cancer Institute.

The first step in this investigation was to ascertain whether wild mice were susceptible to the virus from an inbred strain and, if so, whether they were capable of transmitting it through successive generations. Twenty newborn females were suckled by high mammary tumor strain C3H females throughout the period of lactation. These were the only wild mice exposed to the virus from inbred mice. When they were two to three months of age they were mated to wild stock males and when the litters were born, a newborn inbred mouse from a strain susceptible to the virus was usually added to each litter. These fostered inbred mice served as test animals for the virus in the milk of their wild foster mothers because previous experiments^{1, 15} had shown that inbred mice can transmit the virus in their milk despite their failure to develop tumors.

TABLE 2
OCCURRENCE OF SPONTANEOUS TUMORS IN UNTREATED WILD HOUSE MICE

	No. of mice	Av. age at death	No. auto- p- sied	Av. age	Tumors							
					Mam- mary		Pulmo- nary		Hepatic		Adrenal	
					No. of mice	Average age	No. of mice	Average age	No. of mice	Average age	No. of mice	Average age
		(months)		(months)		(months)		(months)		(months)		(months)
Breeding	36	21	25	23	1	25	5	25	1	24	1	25
Non-breeding	9	20	9	20	0	—	1	19	0	—	0	—

The results of the experiment are presented in TABLE 3. Of the 20 fostered wild mice and 71 descendants, 20, or 11 per cent, developed mammary tumors. This incidence is considerably above the three per cent incidence of mammary tumors shown by the breeding wild mice of TABLE 2. Hormonal stimulation may have been of some importance in the production of mammary tumors in the wild mice of TABLE 3, for eight failed to become pregnant, 64 bore one litter, 16 had two litters, two had three litters, and one had four litters. These groups contained 0, seven, one, one, and one tumor-bearing mice, respectively. After raising their last litter, all wild mice of these experiments were kept in cages that did not contain exercise wheels and it is known¹² that exercise is essential for the achievement of estrus in wild mice. The availability of exercise wheels at all times may have increased the incidence of mammary tumors in the wild mice.

The occurrence of tumors in inbred mice suckled by wild mice was the most significant finding of the experiment. Each of the fostered mice give birth to one litter which was removed soon after birth. Data in TABLE 3 show that of 44 mice, 25, or 57 per cent, developed tumors. This high incidence is significant because the test animals were strain C¹⁶ or virus-free strain C3H¹⁷ mice in which the incidence of mammary tumors is less

than five per cent. Furthermore, none of their non-fostered sisters developed a tumor. It is believed the occurrence of tumors in the fostered inbred mice shows that they obtained the virus in the milk of their wild foster mothers.

A typical result in the descendants of a fostered wild mouse is shown in TABLE 4. Neither the fostered mouse nor any of her four daughters had a tumor, but five of the seven inbred mice suckled by them did develop tumors. In later generations, three of four wild mice in one line developed tumors but all in another line remained tumor-free. Two of three test mice, who were nursed by the tumor-free line developed tumors, however. Such findings show that the wild mice transmitted the virus through four successive generations without developing tumors.

TABLE 3
OCCURRENCE OF MAMMARY TUMORS IN FOSTERED WILD MICE AND IN INBRED MICE SUCKLED BY FOSTERED WILD MICE

Generation	Wild mice					Inbred mice fostered by wild mice				
	Num- ber of mice	Num- ber devel- oped mam- mary tumor	Aver- age age	Num- ber died free of mam- mary tumor	Aver- age age at death	Num- ber of mice	Num- ber devel- oped mam- mary tumor	Aver- age age	Num- ber died free of mam- mary tumor	Aver- age age at death
			(months)		(months)			(months)		(months)
Fostered	20	3	16	17	22	10	5	11	5	20
1	24	2	18	22	18	20	12	12	8	22
2	32	3	16	29	21	8	4	15	4	23
3	10	1	16	9	18	6	4	13	2	26
4	5	1	16	4	11	—	—	—	—	—

The outcome of the experiment indicated that, when wild mice obtained the virus from an inbred strain, it did not produce many tumors in them, but they were able to transmit the virus through four generations.

The next, and probably most interesting, step in this investigation was to determine whether the wild house mouse carries the mammary tumor virus. This experiment consisted of the foster nursing of 15 newborn inbred mice by wild mice throughout the nursing period. Nine of the fostered mice were derived from an agent-free colony of strain C3H mice¹⁷ and six from a colony of strain C.¹⁶ These were suckled by eight wild foster mothers, seven of which (procured from Dr. Schneider) were nursing their first litters. None of the foster mothers developed a mammary tumor.

The 15 fostered mice were the only inbred animals to ingest the milk of wild mice. When they were two to three months of age they were bred to brothers to produce the F₁ generation. In this manner, five generations were procured. The occurrence of mammary tumors in the fostered mice and in their descendants is shown in TABLE 5. Selection in the F₃ genera-

tion accounts for the smaller number of mice. Two lines were selected for further propagation because, at that time, their fostered ancestors had developed tumors.

There were 84 mice in the first three generations and 17, or 20 per cent, had tumors. Each mouse in these generations gave birth to one or two

TABLE 4
OCCURRENCE OF MAMMARY TUMORS IN THE DESCENDANTS OF A WILD MOUSE WHO WAS FOSTERED-NURSED BY A STRAIN C3H MOUSE AND IN INBRED MICE FOSTER-NURSED BY THE WILD MICE*

Generation					
Fostered		$\begin{array}{c} 1 + 11 \\ 2 - 19 \\ 6 + 9 \\ 106 - 31 \end{array}$			
		Fostered by C3H female			
1	$\begin{array}{c} 13 - 23 \\ 123 - 12 \end{array}$	$\begin{array}{c} 9 + 10 \\ 118 - 11 \end{array}$	$\begin{array}{c} 8 + 15 \\ 119 - 26 \end{array}$	$\begin{array}{c} 18 + 11 \\ 138 - 25 \end{array}$	
2	$\begin{array}{c} 29 - 26 \\ 148 - 24 \end{array}$	$\begin{array}{c} 26 + 11 \\ 149 - 24 \end{array}$	$\begin{array}{c} 20 + 12 \\ 145 + 19 \end{array}$	$\begin{array}{c} 180 - 24 \end{array}$	
3	$\begin{array}{c} 45 + 8 \\ 182 - 19 \end{array}$	$\begin{array}{c} 183 - 19 \end{array}$	$\begin{array}{c} 31 + 10 \\ 187 + 16 \end{array}$		
4	$\begin{array}{c} 214 - 9 \end{array}$	$\begin{array}{c} 201 - 10 \end{array}$	$\begin{array}{c} 202 + 16 \end{array}$		

* Numbers above 100 are those of wild mice. Numbers below 100 are those of inbred mice foster-nursed by wild mice and are placed above the number of the foster mother. After the number of each mouse a + sign denotes the occurrence of mammary tumor, - denotes death without tumor. The age in months is given after the + or - symbol.

TABLE 5
OCCURRENCE OF MAMMARY TUMORS IN INBRED MICE FOSTER-NURSED BY WILD MICE, AND IN THEIR DESCENDANTS

Generation	Number of mice	Number developed mammary tumor	Average age	Number died free of mammary tumor	Average age at death	Number living	Average age
			(months)		(months)		(months)
Fostered	15	4	20	11	21	—	—
F ₁	36	4	17	32	21	—	—
F ₂	33	9	18	24	21	—	—
F ₃	7	4	14	3	21	—	—
F ₄	7	5	19	2	21	—	—
F ₅	26	10	15	4	15	12	21

litters. The tumor incidence in these mice was higher than the incidence of less than five per cent in the colonies from which they came, which suggests that the wild mouse milk contained a mammary tumor virus. The tumor incidence and average age at which tumors arose in these mice, when compared with the higher incidence and lower average age of tumor development in mice from the same colonies after exposure to the virus in

strain C3H milk,¹⁸ indicates that the virus harbored by wild mice was low in concentration or activity.

As stated previously, two lines of mice were selected for propagation in the F₃ generation. One of these failed to produce offspring but the other has become established. Consequently, all mice of the F₄ and F₅ generations in TABLE 5 are descendants of one of the fostered mice.

The occurrence of tumors in this family is shown in TABLE 6. Fostered female No. 104 was derived from the strain C colony. Her mother died without a tumor at 20 months of age and her two sisters died tumor-free when 19 months old. Offspring of No. 104 were not included in the colony, but two offspring of a sister died when 15 and 22 months old without developing a mammary tumor. These data indicate that mouse No. 104 did not acquire the virus while a member of the strain C colony.

TABLE 6
OCCURRENCE OF MAMMARY TUMORS IN THE DESCENDANTS OF A STRAIN C
MOUSE WHO WAS FOSTER-NURSED BY A WILD MOUSE*

Generation Fostered	Strain C 104 + 17 Fostered by female No. 2 w											
F ₁	127 — 22 132 — 20 130 — 19 133 — 14 131 — 20 134 + 16											
F ₂	140 — 22 141 — 22 138 — 21 139 + 17 192 — 20 178 + 16 179 — 20 180 — 24 181 + 20											
F ₃	168 + 18 167 + 10 202 + 15											
F ₄	216 — 21 217 + 23 210 + 18 211 + 16 212 — 21 213 + 17 218 + 20											
F ₅	223 L 22 224 L 22 231 — 20 235 L 21 236 L 21 237 L 21 238 — 18 219 + 16 241 L 20 242 + 18 243 L 20 226 + 11 227 + 16 228 L 22 225 + 17 239 — 16 240 + 13 220 + 14 231 + 12 233 L 21 234 + 13 229 L 22 230 — 5 244 L 23 245 + 18 246 L 20											

* After the number of each mouse a + sign denotes the occurrence of mammary tumor, — denotes death without tumor, L denotes living. The age in months is given after the symbol.

The family is now in the F₅ generation and, as shown in TABLE 6, which includes only the first five generations, each generation is showing a relatively high incidence of tumors. This suggests that in mice of strain C the activity or concentration of the virus is becoming enhanced.

According to the findings in this experiment, wild house mice carried a mammary tumor virus. This observation will, of course, have to be confirmed. At present, available data suggest that the wild mice harbor a mammary tumor virus which produces few tumors and is detectable in their milk. Such a virus fulfills the requirements for the virus theory of cancer to a considerable extent, insofar as this type of tumor in this species is concerned.

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OBSERVATIONS ON THE MOUSE MAMMARY CARCINOMA VIRUS*

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The elucidation of the role of viruses in cancer has been remarkably slow. Forty years have elapsed since Rous¹ discovered that fowl sarcoma can be transmitted by cell-free filtrates. In retrospect, however, one perceives that some real progress has been made. This monograph has far greater significance than the fact that it provides a medium for the dissemination of information and ideas. It is significant because it signalizes the transition from the hypothetical assumption of viruses as a cause of cancer to the acceptance of viruses as actually playing a role in cancer.

The time-consuming nature of cancer research, and the experimental hazards involved in it have been largely responsible for the delay, but some of it can certainly be ascribed to the stubborn human reluctance to accept new ideologies. Appreciation of the role of viruses in cancer does not imply that all cancers are viral in origin, although that is a perfectly respectable speculation. It neither throws into discard the correlative factors involved in cancer nor deprecates the importance of hormonal and genetic features. In fact, it is highly probable that additional factors will emerge in the course of research.

Since Bittner's discovery² of the nursing influence, it has been possible to define mouse mammary carcinoma as a disease of the adult female, acquired in infancy through an agent transmitted in the mother's milk. The definition is neither complete nor exclusive, but it is a consequence of Bittner's demonstration of the nursing influence. This laboratory, among others, undertook the task of isolating the agent in the milk, one of the factors in the disease, without prejudice to the other features of the disease. Spherical particles approximately 100 millimicrons in diameter were isolated from the milk of high cancer strain mothers. On administration to young low cancer strain females, these particles produced the disease in a manner similar to that of foster-nursing. It was concluded that these infectious particles constituted the sought-for mammary carcinoma virus, the appellation "virus" having been adopted by general consent.³ These conclusions are in general agreement with the results obtained by other workers, including Andervont and Bryan⁴, Kahler and Bryan,⁵ and Ludwik Gross.⁶ Barnum *et al.*⁷, for example, had fractionated minced breast tumors by centrifugation and produced tumors with the microsome or large particle fraction at one microgram to ten milligram levels. Porter and Thompson⁸ have demonstrated in an elegant manner the presence of particulate bodies in C3H mammary carcinoma tissue cultures. The Leeds group⁹ has also actively pressed the proposition that the milk agent is particulate in nature.

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That group employed somewhat different methods and their findings are at variance with ours in that they obtained much smaller particles than we did. It is interesting, however, that they reported the presence of a few larger particles of about 1200 Å in diameter along with a preponderance of those of about 300 Å.

The details of the isolation have been described elsewhere, but will be recapitulated here in the interest of coherence. The opacity of milk is an indication of the large casein aggregates which had to be removed by one means or another in order to afford the separation of the virus particles by differential centrifugation. Accordingly, these casein aggregates were altered either by enzymatic agglomeration or degradation. The employment of chymotrypsin, hitherto unused in virus isolation, was actually suggested by the natural history of the disease (the infectious agent in nature is transmitted via the gastro-intestinal tract, indicating a probable immunity of the agent to at least some proteolytic systems). This guess was borne out. Chymotrypsin does not impair activity even when it is employed exhaustively and repeatedly (TABLE 1).

TABLE 1
BIOLOGICAL ACTIVITY OF PARTICLES FROM PARIS RIII MILK

<i>Dose in micrograms N</i>	<i>Enzymatic</i>	<i>Citrate dialysis</i>
1.6	35/60*	4/8
1.0	14/35	7/16
8×10^{-3}	4/16	
6×10^{-4}	0/14	
8×10^{-7}	0/19	

* Number of tumors.
Number of animals.

Trypsin, on the other hand, inactivates the material rapidly. The chymotrypsin-treated specimen, with the activity indicated in TABLE 1, was treated additionally with crystalline trypsin for one-half hour and then tested by administration of ten times the previous dose. No tumors resulted in 51 animals. Passey *et al.* did report, however, that tumors were produced by material which had been desiccated and treated with trypsin. A word of warning might be appropriate here. The first specimen of chymotrypsin employed was obtained from Dr. M. Kunitz of the Rockefeller Institute. It has not been matched in activity by commercial specimens ostensibly prepared by the same method. There was some evidence that commercial preparations of chymotrypsin are not always free of trypsin.

Similarly clean particle suspensions were prepared by an alternative method. It was argued that casein could be disaggregated by decalcification, and so whole milk was dialysed against volumes of sodium citrate solution. The particles obtained after rather uncomplicated differential centrifugation produced tumors as shown by TABLE 1. The results by decalcification warrant two conclusions: (1) that calcium is not essential to this virus activity or that calcium is not readily removed from the particle;

and (2) that carcinogenic activity probably does not reside in low molecular weight substances adventitiously adsorbed on the particles. An improved decalcification procedure which simplifies the method of segregation of the mammary carcinoma agent from milk¹⁰ has recently been developed in another laboratory. It is based on the use of a cation exchange resin (Dowex 50), which, when added to milk, solubilizes casein by removal of calcium from the complex molecule of calcium casein phosphate. According to McCarty and Gross, large quantities of the agent were segregated from as little as one ml. of milk.

There has been very little opportunity to study the chemical composition of the particles. It was reported earlier that the virus particles contain nucleic acid, that is, alkaline extracts gave the absorption spectrum typical of nucleic acid. All attempts to isolate the nucleic acid by conventional methods have failed, indicating that it is more firmly bound to protein than is the case with animal tissues, an observation all too well borne out by previous experience with the nucleic acids of animal viruses, notably that

TABLE 2

	RNA DNA
Virus particles.....	4.8
Whole <i>tetrahymena geleii</i>	9.6
Whole mouse liver.....	5.7
Whole carcinoma 755.....	1.1
Liver nuclei.....	1.4
Carcinoma 755 nuclei.....	0.3

of Knight with influenza virus.¹¹ It is anticipated, however, that methods now under development will aid in characterizing the nucleic acid component of the particles. When colorimetric carbohydrate assay methods, for example, were applied to the material, it was found that both pentose and desoxypentose sugars were present in the particle in the approximate ratio of 4.8 parts pentose to one part desoxypentose. This ratio can be compared with similarly derived ratios in other tissues (TABLE 2). The relative proportion of the two nucleic acids in the particles is therefore neither distinctive nor significant.

Our earlier preparations from milk were found, both ultracentrifugally and electrophoretically, to contain two components. Attempts to partition the biological activity by means of those techniques were failures and all components produced tumors in high yield. Our more recent preparations, on the other hand, exhibit only one component in the ultracentrifuge and in the electrophoretic cell. Gradient experiments with the earlier preparations in sucrose were also equivocal and the low tumor yields throughout the gradient system indicated that the high concentrations of sucrose employed therein were not altogether innocuous. The experiments did indicate, however, that the specific gravity of the particles was greater than 1.15. The experiments are being repeated with other media. Revised physical constants and properties will be reported elsewhere.

It has not been possible to establish virus activity on a quantitative basis as a practical measure, possibly because of the other factors involved in the disease. One of the most intriguing aspects of the disease, for example, is that the transmission of the agent must be accomplished when the recipients are very young, if they are to develop tumors in adult life. This fact is tempered further by the fact that chronological age is often at variance with developmental age. On the other hand, there is considerable presumptive evidence that the virus begins to multiply or attain tumorigenic activity some time after maturity is reached. Bittner¹² once suggested that the milk agent might actually develop between the first and second litters, or even between the second and third. It was possibly fortunate for our experiments that the donor Paris mice were milked after their third litters, and it was optimal also from the standpoint of good dairy practice. Although lactation in itself probably plays an important part in viral proliferation as well as in virus transmission, it has no bearing on the cancer incidence of the adult mouse, since in the Paris R3 strain the incidence is just as high in virgin as in bred mice.¹³

Some considerable attention has been paid in the past to the occurrence of the infective agent in tissues other than the lactating breast. The agent has been found in tumors, blood, and a number of other organs. Our failure to produce a cancer-free strain of Paris R3 mice by Caesarean section suggests that the agent is also present in the pregnant uterus. Of 15 females so delivered and brought to maturity, five developed tumors. The agent, then, is introduced early in life, in some instances possibly to the embryo, but largely through nursing. It appears later in various organs, multiplies, and appears in abundance in the lactating breast. The interval between access to the host and multiplication of the virus or appearance of cancer, the so-called latent period, is yet to be explored. One relevant experiment on this phase consisted in the preparation of suspensions of mammary gland tissue from two-months-old virgin Paris females and inoculation of C57 test mice with the equivalents of one breast per mouse. Not one tumor was obtained in 100 mice. This was a test of infectivity. No microscopic search for particles was made. Andervont¹ has suggested that mice delivered by Caesarean section might die free of tumors but yet pass on an agent which could produce tumors in later generations. Bittner¹⁴ noted that low-cancer-strain mice fostered by high-tumor-strain females might not necessarily develop tumors, but can transmit the influence in the milk. Bittner further suggested that the milk of mice might contain an "inactive" influence which might become either "active" or arise *de novo*. There may be a pre-viral or inactive viral stage, therefore, followed by activation to the true cancer-producing virus. This possibility is indicated by our experience with the particles from foster-nursed C57 milk. C57 mice were foster-nursed on Paris R3 mothers, and on their maturity were milked after their first pregnancy. This milking may have been unwittingly hasty in view of the foregoing considerations on the time of maximal activity. At any rate, particles very similar to those obtained from the Paris milk, perhaps a little less in average diameter, were found in abundance. Suspensions of these particles were injected into 65 young C57

mice. No tumors were obtained. Apparently these particles require activation. It is necessary to await the next generation in order to support the concept of activation.

Immunological specificity is an important consideration in cancer and considerable work has been reported from other laboratories on the immunological behavior of milk agent preparations. Andervont and Bryan,¹⁵ and Green and Bittner¹⁶ have shown that the agent was able to stimulate the production of neutralising antibodies, and that the agent is antigenically different from mouse tissues. In view of the importance of the subject, very considerable emphasis was placed on a quantitative study of the immunological properties of the purified virus particles. These experiments will be reported in detail elsewhere.¹⁷ In summary, however, it was found in those experiments that the particles from the Paris R3 milk were strongly antigenic, especially when adjuvants were employed. Exhaustive adsorption of anti-particle sera by means of C57 milk proteins and by C57 breast tissue extract left one-half to almost all of the anti-particle nitrogen still in a form precipitable by particles. Cross reactivity of the rabbit anti-particle serum to C57 mouse milk serum proteins varied in different sera but was never extensive. Antibody to antigen ratios were found to be low, often less than one, unlike those usually found with smaller antigens. The large particle size, estimated to be roughly 300 times 10^6 , possibly has some bearing on this ratio. These experiments are consistent with the earlier view that the specificity of the milk agent is different from that of soluble proteins occurring ordinarily in the milk and breast tissue of the normal mouse. Proteins of other mouse tissues must be tested before we can decide whether or not the particle contains host components as well as specific viral components. Experiments are under way to determine whether or not antibodies to the particles actually exert a neutralizing effect on the carcinogenicity of the particles, or their ability to reproduce in the host.

Summary

Spherical particles with high density to the electron beam have been isolated in quantity from the milk of high cancer strain mice. These particles transmit the disease in high dilution in characteristic manner. These particles elicited antibodies in the rabbit, and were shown by immunochemical techniques to be antigenically distinct from normal proteins of the mouse or mouse milk. It is concluded that these particles constitute the virus responsible for mouse mammary carcinoma.

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THE SIGNIFICANCE OF PARTICLES IN HUMAN MILK*

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It has been known for some time that mammary carcinoma in mice is caused by a filterable agent which is transmitted from one generation to another through the milk of nursing females.¹⁻³ Recently, small spherical particles having a diameter varying from 20 to 200 m μ have been visualized, with the aid of an electron microscope, in samples of milk obtained from nursing mice known to carry the mammary carcinoma agent.^{4, 5} Similar spherical particles have also been found in tumor extracts⁴⁻⁹ or in cells cultured¹⁰ from mouse mammary carcinomas.

It is difficult, at the present time, to form an opinion concerning the nature of these particles. One has to take into consideration that particles similar in shape and size have been found also in extracts prepared from presumably normal mouse tissue organs,^{5, 6, 8-10} and frequently also in milk samples collected from mice apparently free from the mammary carcinoma agent.^{5, 6, 8, 11} The differentiation of the various particles observed in the electron microscope is, at the present time, quite difficult. Many particles which may be of an entirely different origin may appear alike to the critical observer. This is particularly true when particles are observed after they have been shadowed with heavy metals.

It is true that the exact nature of the particles detected in mouse milk has not yet been determined. Their consistent presence, however, in mouse milk samples known to contain the mammary carcinoma agent, and their only occasional presence in samples of milk collected from mice known to be free from the tumor agent suggests that they may represent the mouse mammary carcinoma agent. Such an assumption was strengthened by the results of experiments recently performed, in which, after selective centrifugation, fractions of samples containing the particles were injected into susceptible mice and produced mammary carcinomas.^{7, 8}

Since the possibility cannot be excluded that at least certain forms of human breast cancer may also be caused by factors similar to those responsible for the development of mammary carcinoma in mice,^{12, 13} a study has been carried out with the purpose of determining whether spherical particles, similar to those found in mouse milk, would also be found in human milk samples, particularly in those collected from women having a family record of breast cancer.¹⁴ In addition, milk samples were also collected from women having a family record of malignant tumors other than that of the breast. Control samples were collected from women having a family record apparently negative for any malignant tumors for two preceding generations. It must be stated, however, that the information con-

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cerning the cause of death of the grandparents of the nursing women frequently was not reliable. It is entirely possible, therefore, that among the presumably negative "control" samples, some might have been collected from women having a family record of cancer among their ancestors. In contrast, information concerning the "positive" samples, *i.e.*, those collected from women having a family record of cancer of the breast or that of other organs, could be considered reliable in practically all instances.*

Materials and Methods

Human Milk Samples. Young, healthy women who had delivered two to seven days previously and were nursing at the time, were interviewed as to the occurrence of malignant tumors in members of their respective families. The women serving as donors were divided into three groups. In group A, twelve milk samples were collected from nursing healthy women whose sisters, mothers or grandmothers had breast cancer. In group B, thirteen samples were collected from nursing healthy women having a family record of a malignant tumor of any type on either their mothers' or fathers' side. In group C, seventy-one control samples were collected from nursing healthy women with a family record apparently free from malignant tumors for two preceding generations. The milk was collected with the aid of a breast pump and placed in a sterile tube. Each sample was collected from a different donor. Fifteen ml. were used for each segregation.

Mouse Milk Samples. Nursing female mice of the C3H inbred line, whose milk was known to contain the mammary carcinoma agent, were used as donors of the "positive" mouse milk samples. Control mouse milk samples were obtained from nursing female mice, presumably free from the mammary carcinoma agent, of the following lines: (a) foster-nursed C3H(f);¹⁵ (b) C57 (black); and (c) white-footed deer mice† (*Peromyscus leucopus noveboracensis*).¹⁶

The mouse milk samples were collected in either of the following ways:

(a) Suckling infant mice two to eight days old were sacrificed by ether inhalation and the cheese-like contents of their stomachs were removed aseptically and placed in a sterile centrifuge tube containing 12.5 ml. of McIlvaine's standard (citrate)¹⁷ buffer pH 7.4. Three to six infants were used, giving a total of 0.1 to 0.5 gms. of milk curd for each individual sample. This was dispersed in buffer by aspiration through a 22 gauge needle, using a sterile 10 ml. syringe.

(b) Nursing female mice were separated from their suckling offspring for 24 hours and, after this lapse of time, were sacrificed by ether inhalation; the breast tissue was aseptically removed, placed in a sterile centrifuge tube containing 12.5 ml. of citrate buffer, and the tube was shaken vigorously to disperse the milk. The breast tissue was then removed by lifting it gently with a sterile needle, leaving a solution of dispersed milk.

* Most of the milk samples were obtained from the New York Lying-In Hospital. A few samples were obtained through the courtesy of private physicians.

† The white-footed deer mice have been used by one of the authors for a research project unrelated to the present study.¹⁶ These mice were obtained from Mr. V. Schwentker, Tumblebrook Farm, Brant Lake, New York, in the Adirondack Mountains. They were trapped at Brant Lake in 1947, and have been bred since that time, mostly by brother to sister mating. Over 200 females more than a year old have been observed in this laboratory; only one of them developed spontaneously a mammary carcinoma.

Segregation of Milk Samples. The preparation of milk samples for electron microscopy involved the elimination of carbohydrate, fat and proteins. This was at first accomplished by either the hydrolysis of the interfering proteins using enzymes, followed by differential centrifugation (Method I),¹⁴ or by the use of a high specific gravity solvent, followed by differential centrifugation (method II).¹⁴ Method I depended upon the complete hydrolysis of molecules of the interfering protein present in the milk. Method II, while giving better results for electron microscopy than Method I, was

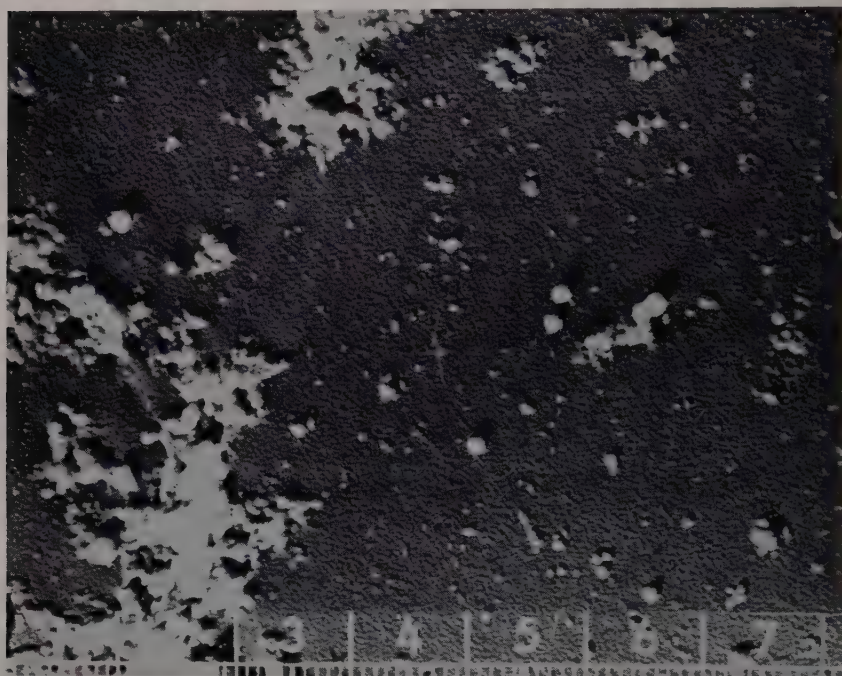


FIGURE 1. Electron-micrograph of colloidal calcium-casein-phosphate suspension, as found in untreated raw cow milk. Numerous calcium-casein-phosphate particles present, many of them spherical, varying in size. Chromium shadowed. 15,000 \times .

complex, and involved the use of a toxic solvent. A simpler, and much more direct approach to the problem was worked out in subsequent experiments,¹⁸ and designated as Method III.

Method III. In the milk, the colloidal calcium-casein-phosphate complex exists as a suspension, which, when examined with the aid of the electron microscope, presents the picture of small, irregular, and sometimes spherical particles¹⁹ varied in size and extent (FIGURE 1). These particles may be readily confused, particularly in shadowed electron micrographs, with other particles of different origin but which are similar in size and form and which may also be present in such samples. It was found that this complex of calcium-casein-phosphate could be reduced to a solution by removing calcium, and replacing it with sodium. This was accomplished

by adding the ion exchange resin "Dowex # 50" (Dow Chemical Company), 20 to 50 mesh, in the sodium cycle, to the milk samples. This procedure removed calcium by replacing it with an equal quantity of sodium, but it did not alter the ionic concentration of the solution. The resin, being inert, insoluble, and of large grain size, could then be readily removed. The electron microscopic examination of the milk samples processed by this method did not reveal the presence of the calcium casein particles, indicating thereby that the calcium-casein-phosphate was reduced from colloidal suspensions into a solution (FIGURE 2).



FIGURE 2. Electron-micrograph of a raw cow milk sample that had been treated with Dowex 50, reducing the calcium-casein-phosphate complex into a solution. Segregated by Method III. Note absence of calcium-casein-phosphate particles. Chromium shadowed. 15,000 X.

The technical procedure was as follows: 15 ml. of whole human breast milk, or mouse milk dispersion, was centrifuged at 750 g. (2,300 RPM) in an angle head (56°) for ten minutes to remove most of the fat. The pellicle of fat formed on the surface was discarded because it has been shown previously that the fat fraction of milk does not contain the mammary carcinoma agent in any appreciable quantity.^{7, 20}

Twelve and a half ml. of this partially fat-free milk was then mixed with two gms. of Dowex 50 (which has been previously thoroughly washed successively with triple distilled water, then with citrate buffer pH 7.4, and allowed to remain wet in this medium). This mixture was agitated vigorously for five minutes and allowed to settle. The sediment was discarded

and the supernatant milk (12.5 ml.) was then mixed with an additional two gms. of Dowex 50. This mixture was agitated for five minutes, once more allowed to settle, and again the sedimented Dowex was discarded. The supernatant decalcified milk was then checked, using either a spectrometer or a very sensitive calcium color test,²¹ to determine whether any trace of calcium was still present. Whenever traces of calcium were still found in the samples, the procedure with Dowex was repeated until the milk was found to be calcium-free. The decalcified and partially fat-free milk was then centrifuged in a Spinco ultra-centrifuge, using an angle head (26°), at 144,700 g. (40,000 RPM) for two hours. A fat pellicle formed at the top of the tube and a sediment settled in the form of a small, opalescent pellet on the bottom of the tube. The supernatant was carefully withdrawn with a syringe and discarded. The pellet was resuspended by aspiration in 12.5

TABLE 1

ELECTRON MICROSCOPIC EXAMINATION OF MILK SAMPLES COLLECTED FROM HEALTHY, NURSING WOMEN HAVING A FAMILY RECORD OF BREAST CANCER

Method of segregation	Number of samples examined	Results of electron microscopic examination. (presence of particles)			
		++	+	±	Negative
I	5	2	3	0	0
II	5	3	2	0	0
III	2	1	1	0	0
Total	12	6	6	0	0

ml. of citrate buffer and recentrifuged at 144,700 g. for two hours. The new pellet resulting from this centrifugation was resuspended in two ml. of 2 per cent ammonium acetate buffer at pH 7 and one drop of this suspension was then placed on an electron microscope grid and air dried. Ammonium acetate buffer was chosen because it is volatile and does not form crystals that would interfere with electron microscopy.

Electron Microscopic Examination of Milk Samples. A preliminary examination was then made with the RCA EMU-2B electron microscope.* After the preliminary examination of the unshadowed sample, the grids were placed in a SC-3 vacuum evaporator (Opt. Film Engineering Co.), and then shadowed at an angle of 15° from a distance of 10 cm., using 20–25 mg. of chromium, in a vacuum of 10⁻⁴ to 10⁻⁵ mm. of mercury. The shadowed samples were then examined in the electron microscope, at least 50 fields being examined of each sample. If particles were present in the preliminary sample after shadowing, electron micrographs were taken at a magnification of 5,000 ×. If, however, no particles were found in the preliminary sample, even after examination at top magnification in order to detect whether some single particle might have been overlooked and be present in the solution, the entire suspension of the sedimented pellet was then centrifuged at 25,000

* The electron microscopic examination was carried out by K. S. McCarty.

g. for ten minutes directly onto a grid. This grid was then air dried and again examined in the electron microscope. Fifty fields were examined in each sample.

It must be emphasized that the conscientious examination of the many different electron microscopic fields of a given sample and the selection of

TABLE 2
RESULTS OF ELECTRON MICROSCOPIC EXAMINATION OF MILK SAMPLES OBTAINED FROM
WOMEN HAVING A FAMILY HISTORY POSITIVE FOR CANCER OTHER
THAN THAT OF THE BREAST

<i>Donor's record number</i>	<i>Method of segregation</i>	<i>Donor's family history</i>	<i>Results of electron microscopic examination. (presence of particles).</i>
1-B	I	Father had carcinoma of the bladder. Mother's aunt had cancer of the uterus.	++
2-B	II	Grandmother had cancer of stomach.	++
3-B	II	Father had cancer of stomach.	±
4-B	II	Father had cancer of intestines.	++
5-B	II	Grandfather had cancer of throat.	++
6-B	II	Father had carcinoma of throat.	+
7-B	II	Father had cancer of colon.	+
8-B	II	Father had bronchogenic cancer.	+
9-B	II	Mother had metastatic carcinoma, primary, undetermined.	+
10-B	II	Father had cancer of lung.	+
11-B	III	Maternal uncle had cancer—site unknown.	+
12-B	III	Grandfather had cancer of liver.	Negative
13-B	III	Father had cancer of stomach.	+

TABLE 3
ELECTRON MICROSCOPIC EXAMINATION OF MILK SAMPLES COLLECTED FROM HEALTHY,
NURSING WOMEN HAVING A FAMILY RECORD PRESUMABLY FREE FROM CANCER

<i>Method of segregation</i>	<i>Number of samples examined</i>	<i>Results of electron microscopic examination. (presence of particles)</i>			
		++	+	±	Negative
I	9	4	1	4	0
II	38	3	8	20	7
III	24	4	9	8	3
Total	71	11	18	32	10

what is presumed to be a "typical" field for the photographs is one of the most important and time-consuming parts of the procedure. It goes without saying that the selection for photography of a "typical" field in a given sample is open to subjective judgment and, in certain samples at least, the decision as to which field is the most representative of the sample is most difficult. Although a perfectly satisfactory technic has not yet been de-

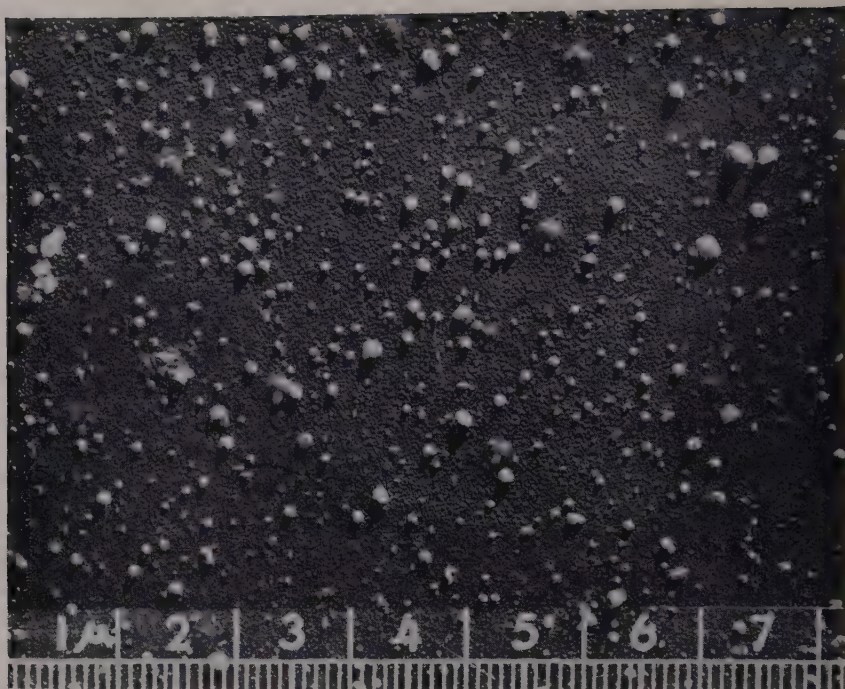


FIGURE 3. Electron-micrograph of a milk sample collected from a healthy woman whose mother had cancer of the breast. Segregated by Method III. Numerous spherical particles ("2 plus"). Chromium shadowed. 15,000 \times .

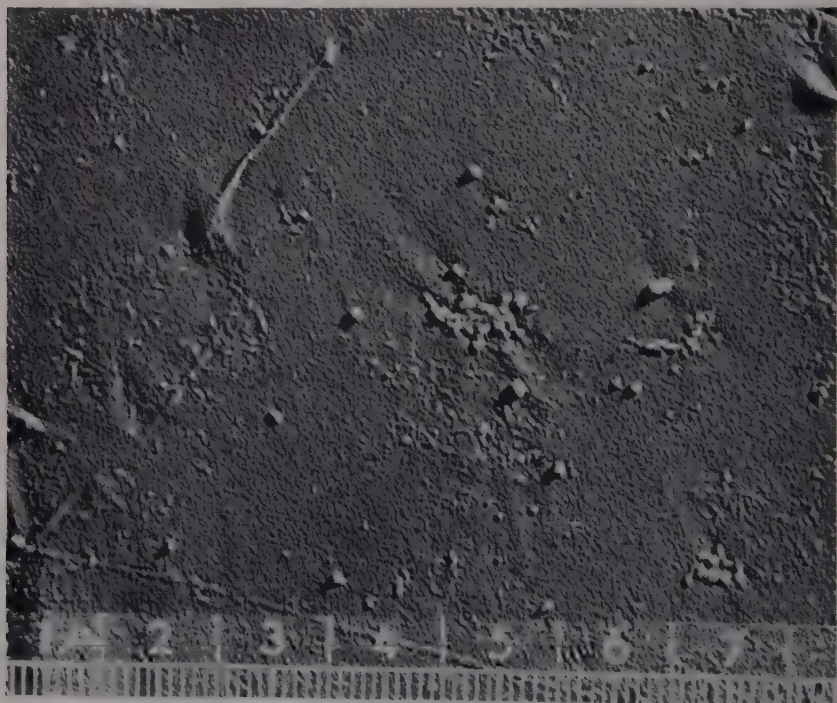


FIGURE 4. Electron-micrograph of a milk sample collected from a healthy woman whose father died of cancer of the stomach. Spherical particles present in all electron microscopic fields examined, but not very numerous ("1 plus"). Segregated by Method III. Chromium shadowed. 15,000 \times .

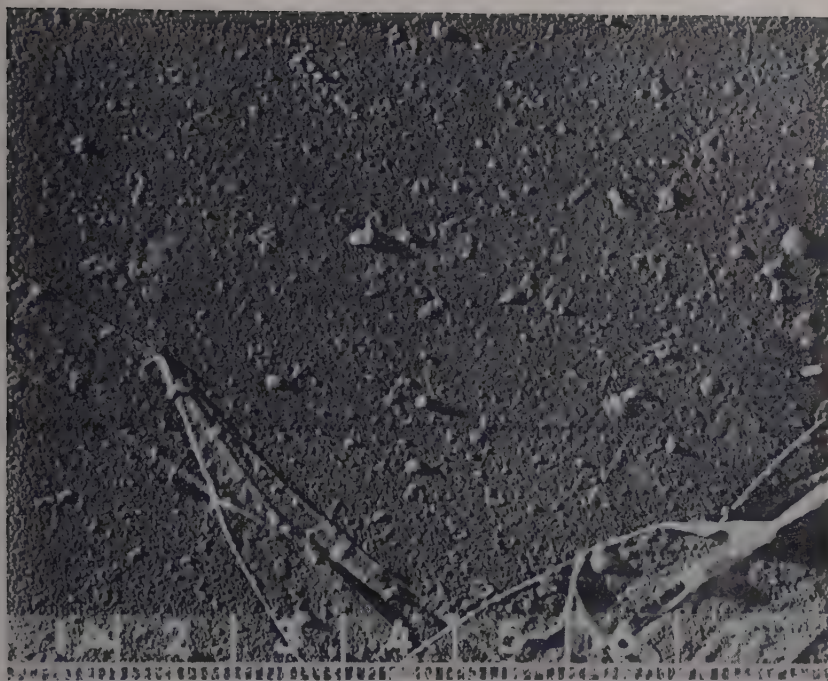


FIGURE 5. Electron-micrograph of a milk sample collected from a healthy woman who had a family record presumably negative for any malignant tumors for two preceding generations. Segregated by Method III. Particles present, though not in large numbers ("1 plus"). Chromium shadowed. 15,000 \times .

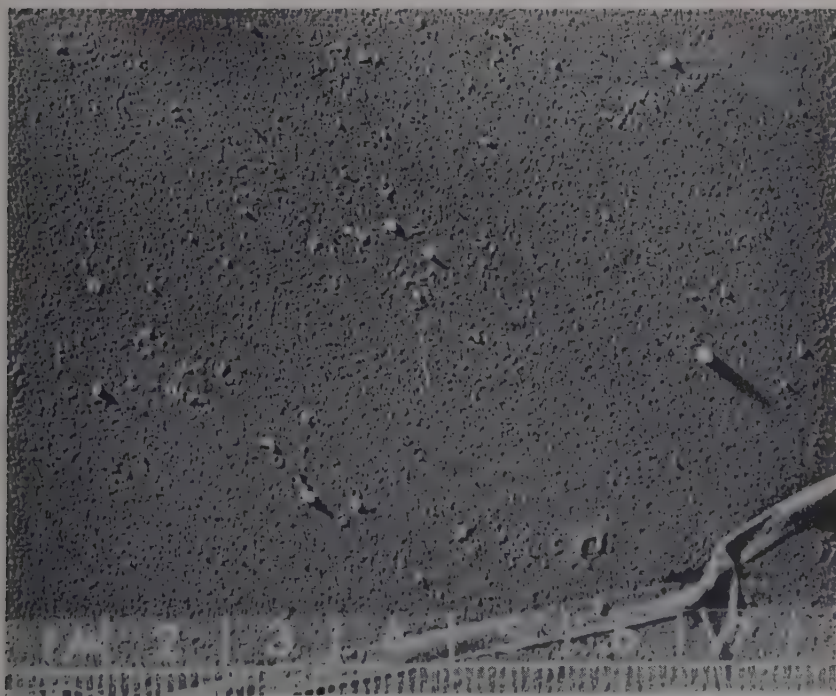


FIGURE 6. Electron-micrograph of a milk sample collected from a healthy woman who had a family record presumably negative for any malignant tumors for two preceding generations. Segregated by Method III. Only occasional, isolated particles found in some of the electron microscopic fields ("questionable"). Chromium shadowed. 15,000 \times .

terminated in our experiments thus far performed, the centrifugation of the samples containing small numbers of the particles directly on the electron microscope grids appears to offer the best assurance that small quantities of particles may be detected in questionable specimens.

Results of Electron Microscopic Examination of Human Milk Samples. The results of the electron microscopic examination of all three categories of human milk samples, segregated by the three methods, are summarized in TABLES 1, 2, and 3. It is evident from these data that all 12 samples collected from women having a family record of breast cancer (Group A, TABLE 1) contained either substantial or large quantities of spherical particles. Similar, although less consistent results were obtained with samples collected from women having a family record of other types of cancers (Group B, TABLE 2).

The particles found in human milk samples had a spherical shape and a diameter varying from 20 to 200 $m\mu$. Their surface was smooth in most

TABLE 4
ELECTRON MICROSCOPIC EXAMINATION OF MILK SAMPLES COLLECTED FROM C3H FEMALE MICE (KNOWN TO CARRY THE MAMMARY CARCINOMA AGENT)

Method of segregation	Number of samples examined	Results of electron microscopic examination. (presence of particles)			
		++	+	±	Negative
I	1	1	0	0	0
II	10	8	2	0	0
III	23	23	0	0	0
Total.....	34	32	2	0	0

instances. When examined before shadowing, they had a high density to the electron beam. After shadowing with chromium at an angle of 15° , they had sharp-edged, long shadows (FIGURES 3 and 4).

In the examination of 71 milk samples collected from women having a presumably negative family record for cancer (Group C, TABLE 3), particles were found only in 29 of them (FIGURE 5); 32 samples had only occasional, single particles in some of the electron microscopic fields (FIGURE 6), and ten samples appeared to be free from any particles at all, but contained some unidentified debris.

Results of Electron Microscopic Examination of Mouse Milk Samples. The electron microscopic examination of "positive" mouse milk samples (collected from C3H mice), using either of the three methods of segregation, demonstrated the presence of spherical particles having a diameter of 20 to 200 $m\mu$ (FIGURES 7 and 8) in all samples examined (TABLE 4). This observation was consistent with those reported previously by Graff^{4, 7} and his co-workers, and also by Passey^{5, 6, 8} and his associates.

Milk samples obtained directly from the breast glands of lactating C3H female mice appeared to have substantially larger quantities of particles than those obtained from the stomachs of suckling C3H infants.

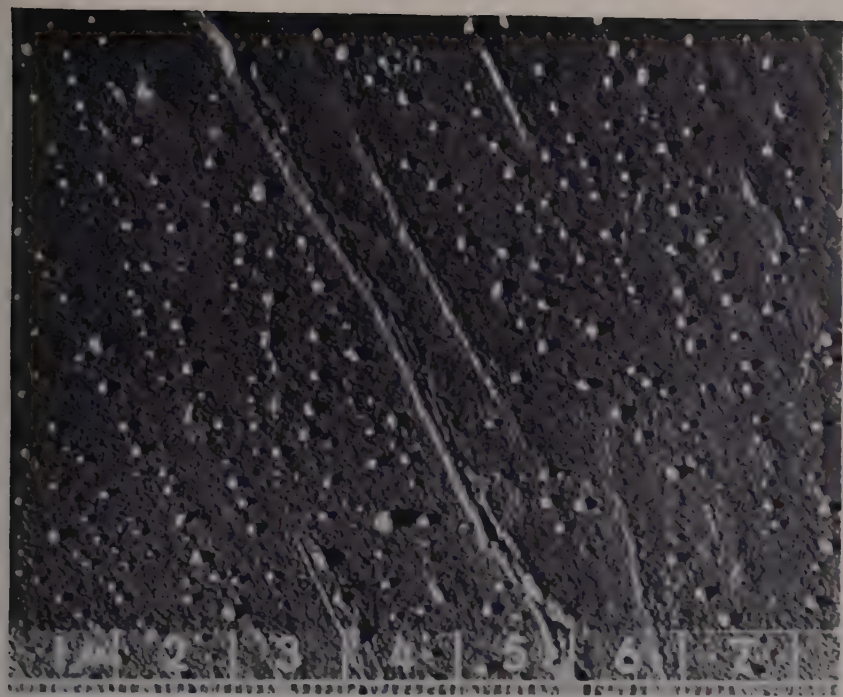


FIGURE 7. Electron micrograph of a mouse milk sample collected from the stomachs of suckling infant mice of the C3H line, known to contain the mouse mammary carcinoma agent. Segregated by Method III. Numerous spherical particles present "2 plus". Chromium shadowed. 15,000 X.

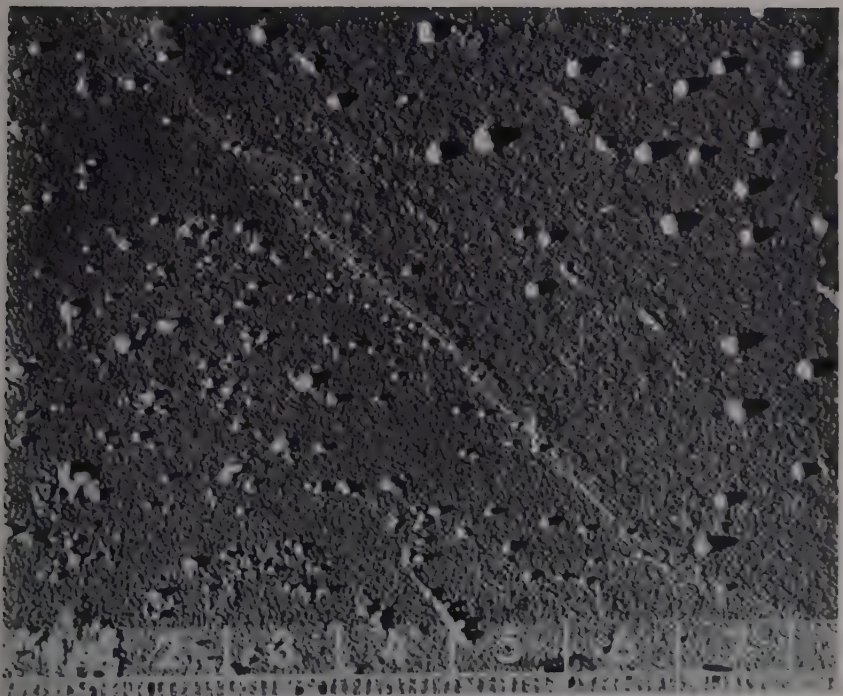


FIGURE 8. Another electron micrograph of a milk sample collected from stomachs of suckling infant mice of the C3H line, known to carry the mouse mammary carcinoma agent. Segregated by Method III. Numerous spherical particles ("2 plus"). Diameter of particles in this electron microscopic field vary from 20 to 200 m μ . Chromium shadowed. 15,000 X.

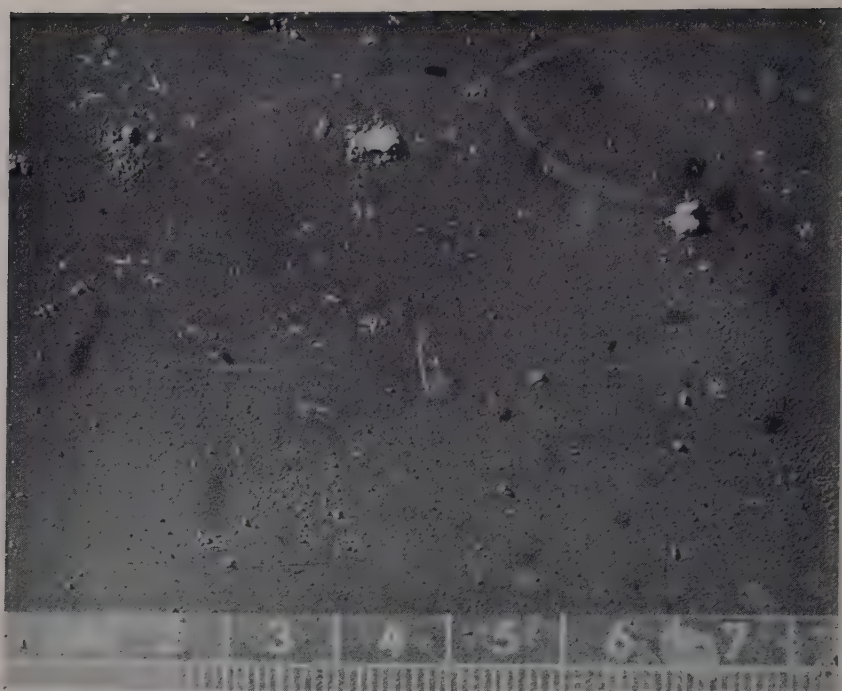


FIGURE 9. Electron-micrograph of milk sample obtained from stomachs of suckling infant mice of a foster nursed C3H(f) subline known to have a very low incidence of "spontaneous" mammary carcinoma. Segregated by Method III. Only occasional isolated particles in some of the electron microscopic fields ("questionable"). Chromium shadowed. 15,000 X.

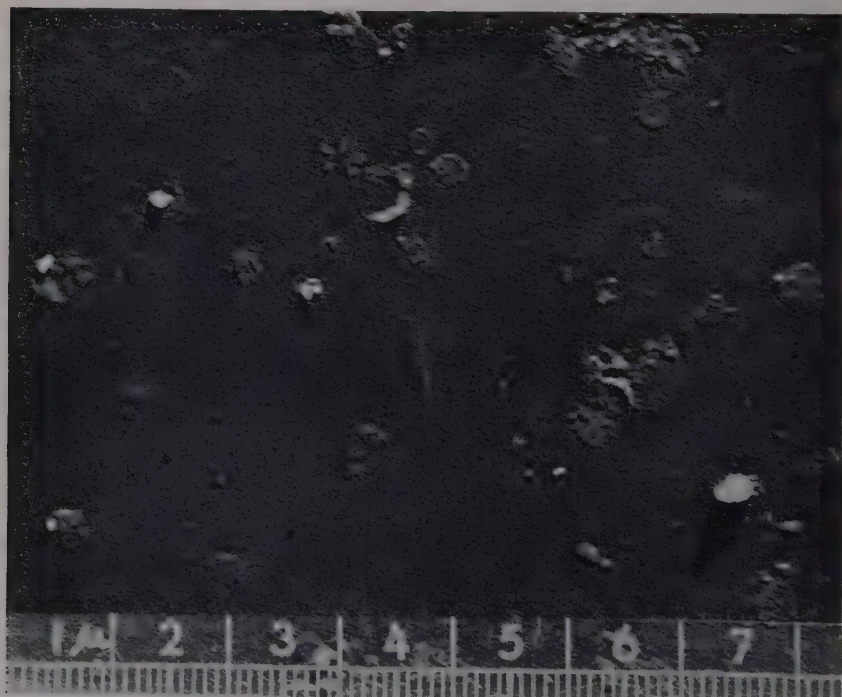


FIGURE 10. Electron-micrograph of a milk sample collected from stomachs of suckling infant mice of the C37 (black) line known to have a very low incidence of spontaneous mammary carcinoma. Segregated by Method III. Spherical particles essentially absent; only a few isolated particles found in some of the electron microscopic fields examined ("questionable"). Chromium shadowed. 15,000 X.

When samples collected from mice presumably free from the mammary carcinoma agent were examined, spherical particles were found much less frequently (FIGURES 9, 10). Many of these samples contained very small

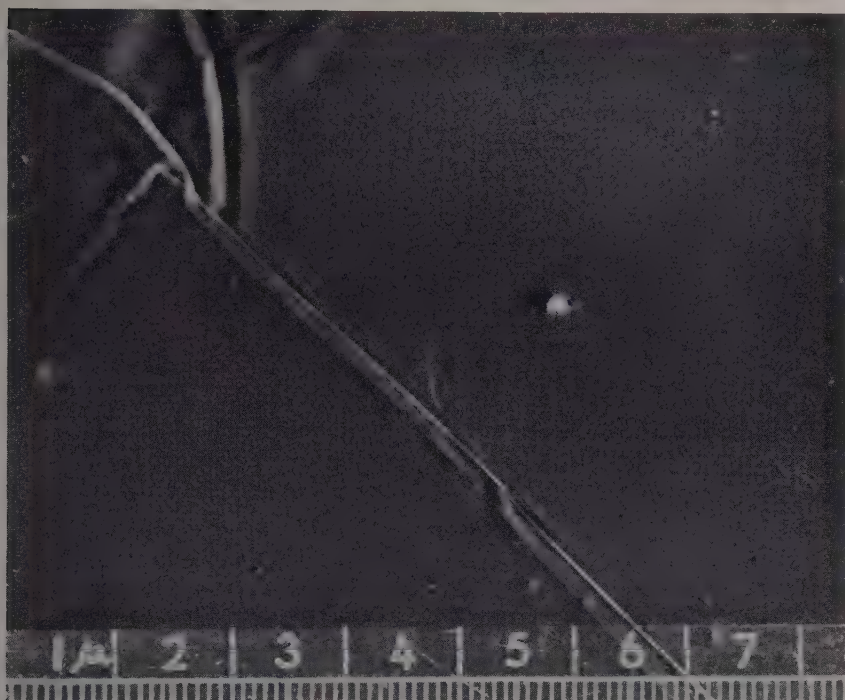


FIGURE 11. Electron-micrograph of a milk sample obtained from stomachs of suckling infant White Footed Deer Mice presumably free from spontaneous mammary carcinoma. Segregated by Method III. No spherical particles found in any of the electron microscopic fields examined ("negative"). Chromium shadowed. 15,000 \times .

TABLE 5
ELECTRON MICROSCOPIC EXAMINATION OF MILK SAMPLES COLLECTED FROM FOSTER NURSED C3H(F) MICE PRESUMABLY FREE FROM THE MAMMARY CARCINOMA AGENT

Method of segregation	Number of samples examined	Results of electron microscopic examination. (presence of particles)			
		++	+	\pm	Negative
II	11	2	6	3	0
III	2	0	0	2	0
Total.....	13	2	6	5	0

numbers of particles in only some of the electron microscopic fields examined and, when Method III of segregation was applied, nine of the 22 milk samples collected from white-footed deer mice appeared to be free from any particles at all of the 20 to 200 $m\mu$ size (TABLES 5-7, figure 11).

Results of Electron Microscopic Examination of Raw Cow Milk Samples. When untreated raw cow milk was examined with the aid of the electron microscope, particles of varying shapes and diameters could be consistently observed (FIGURE 1). Most of these particles, however, represented calcium caseinate.¹⁹ In order to eliminate the calcium casein particles, the raw cow milk samples were processed exactly like the human and mouse specimens by Method III, using Dowex 50 for decalcification. Of 22 cow milk samples, segregated by this method, only three were found to contain a few

TABLE 6

ELECTRON MICROSCOPIC EXAMINATION OF MILK SAMPLES COLLECTED FROM C57 (BLACK) MICE PRESUMABLY FREE FROM THE MAMMARY CARCINOMA AGENT

Method of segregation	Number of samples examined	Results of electron microscopic examination. (presence of particles)			
		++	+	±	Negative
I	1	0	1	0	0
II	7	4	1	2	0
III	5	0	0	5	0
Total.....	13	4	2	7	0

TABLE 7

ELECTRON MICROSCOPIC EXAMINATION OF MILK SAMPLES COLLECTED FROM WHITE-FOOTED DEER MICE PRESUMABLY FREE FROM THE MAMMARY CARCINOMA AGENT

Method of segregation	Number of samples examined	Results of electron microscopic examination. (presence of particles)			
		++	+	±	Negative
II	2	1	0	1	0
III	22	0	2	11	9
Total.....	24	1	2	12	9

particles in most of the electron microscope fields examined, 12 were "questionable," showing here and there a single particle in some of the fields only, and seven were completely negative.

Discussion

The results of experiments reported in this paper suggest that human milk samples frequently contain small spherical particles ranging in size from 20 to 200 m μ having a high density to the electron beam, which can be detected with the aid of an electron microscope. Although many human samples taken at random may contain such particles, it was found that they were present with few exceptions in either large or fairly large quantities in samples collected from milk of women having a history of malignant tumors in their family, particularly in those obtained from women having a family

record of breast cancer. The examination of samples collected from women having a family record presumably free from cancer revealed the presence of particles in large or fairly large quantities in some instances. Frequently, the particles were present in such samples in small quantities only. In a few samples, no particles were found at all.

Similar findings were reported by Passey and his co-workers^{22, 23} at the University of Leeds, England. The British authors have also examined human milk samples, as well as extracts prepared from human breast cancer. They were able to obtain for their study four human milk samples collected from women suffering from breast cancer. In three of the four samples, there were numerous particles present. In the fourth sample, particles were also present, though fewer in number. When 12 milk samples were examined from nursing women who were in good health, one was found to be completely free from any particles and, of the remaining samples, one had numerous particles, four had fairly numerous particles, and six had only occasional particles. The results obtained by the British authors appear to be consistent with those obtained in our laboratory, even though their method of segregation of milk samples was different from those used in our study. The smaller size of the particles described by the British investigators may be due to a difference in the technic of segregation.

Examination of mouse milk samples with the electron microscope gave results which were essentially similar to those previously obtained by Graff *et al*,^{4, 7} and also by Passey and his associates.^{5, 6, 8} Thus, spherical particles of a high density to the electron beam and measuring 20 to 200 $m\mu$ in diameter were found in the mouse milk samples known to contain the mouse mammary carcinoma agent, although milk samples collected from mice known to be free from the tumor agent contained only occasionally small quantities of particles, or, at least in some instances, no particles at all.

At the present time, it does not appear possible to offer any conclusive interpretation of the possible nature of the spherical particles observed with the electron microscope in human milk samples. The investigator is confronted with the fact that, although such particles appear to be quite consistently present in human breast cancer cells²⁴ or in tumor extracts,^{23, 25} as well as in milk samples collected from women having a family record of cancer,^{22, 14, 23} similar particles, though less frequently and in lesser quantities, can be frequently detected also in milk samples collected from women having a family record presumably free from cancer, as well as in various biological samples and tissue extracts apparently free from tumors. Further studies are necessary to clarify the nature of these particles and to differentiate among the possibly different kinds of particles present in tissue extracts and various biological samples, including milk.

It should be emphasized that the method of preparation of the milk samples for electron microscopy is of major importance. Thus, if calcium is not completely removed from the milk sample, the colloidal calcium-casein-phosphate, always present in untreated milk, may be seen, when examined with the electron microscope, in the form of small particles (FIGURE 1). The following points may be helpful in their identification: their size varies considerably, depending upon the method of preparation. Some of the

particles may be spherical, but their edges are not always smooth. When examined prior to shadowing, they do not show any appreciable density to the electron beam. After shadowing with chromium, they frequently show a dent on their surface. Their shadows are rather short, and have edges which are frequently not smooth. This latter phenomenon may be caused by the fact that these calcium-casein-phosphate particles are rather shallow, shallow, and flatten out on drying.

Although Method III was used for segregation, assuring the complete removal of calcium from the samples, particles which evidently did not represent calcium caseinate could still be found, not only in some of the presumably "negative" control human samples, but also in some of the cow milk samples, as well as among those collected from mice known to be free from the mammary carcinoma agent. The nature of these particles remains obscure. Several explanations are possible. They may be normal components of milk or some of them may represent extraneous viruses, or virus-like agents. Various microbes can be detected with the optical microscope in samples of raw cow milk, and it is only reasonable to assume that submicroscopic organisms, some of them harmless, others possibly pathogenic, may also be occasionally present in raw cow milk as well as in raw milk samples collected from other mammals.

Theoretically, it is possible to assume that at least some of these particles may represent a tumor agent. This possibility is clearly at hand in the case of milk samples collected from mice known to carry the mammary carcinoma agent. In the case of human samples, however, further studies are necessary to clarify the nature of such particles, to determine whether or not they are associated with a hypothetical cancer agent.

The difficulty of properly interpreting the significance of particles in human milk samples was increased by the fact that such particles were found in most of the samples of milk collected from women having a family record of various malignant tumors other than that of the breast, such as carcinoma of the intestinal tract, *etc.* Should the findings on human milk be directly comparable to those resulting from experiments carried out on mice, one would rather have expected the tumor agent to be absent in milk samples obtained from women whose ancestors died from tumors other than that of the breast. Thus, in mice at least, the agent was found to be present in the milk of nursing females having a family record of mammary carcinoma, but has not yet been found in milk samples obtained from female mice whose ancestors died from other tumors. The current method used to determine the presence of the mouse mammary carcinoma agent, however, is that of a biological assay. This consists of inoculating a sample to be tested for the presence of the agent into susceptible animals with the view of determining whether they will develop tumors. It is obvious that such a method is far from reliable, since many tumor agents, even though inoculated into susceptible hosts, may require additional activating conditions to actually reproduce tumors. When such conditions are lacking, even though the agents were inoculated and are present in the host, they may remain dormant.¹⁶

It is possible, therefore, that not only the mammary carcinoma agent, but

also other tumor agents may be eliminated in the milk of lactating females, even though the transmission of such tumor agents does not necessarily have to occur through the milk.¹⁶

Apparently, various viruses and other pathogenic agents may be eliminated in the milk of infected hosts or carriers. The mumps virus was recently isolated from the milk of a woman who developed parotitis two days before delivery, and also from milk of lactating monkeys following intravenous inoculation with the virus.²⁶

Summary and Conclusions

Young healthy women who had delivered two to seven days previously and were nursing at the time were interviewed as to the occurrence of malignant tumors in members of their respective families. The women serving as donors were divided into three groups: In Group A, twelve milk samples were collected from nursing healthy women whose sisters, mothers or grandmothers had breast cancer. In Group B, thirteen samples were collected from nursing healthy women having a family record of a malignant tumor of any type on either their mothers' or fathers' side. In Group C, seventy-one control samples were collected from nursing healthy women with a family record apparently free from malignant tumors for two preceding generations.

All 12 samples collected from women having a family record of breast cancer contained either large or fairly large quantities of particles. Of the 13 milk samples collected from women having a family record of any type of malignant tumor on either paternal or maternal side, 11 contained large or fairly large quantities of particles; one sample contained a minimal number of isolated particles in some of the electron microscopic fields; and one sample did not contain any particles at all. Finally, the examination of 71 milk samples obtained from women having a presumably negative record for cancer revealed either large or fairly large quantities of particles in 29 of them; while 32 contained only occasional particles in some of the electron microscopic fields examined and ten had no particles at all.

The true nature of the spherical particles found in samples of human milk remains obscure. They may be biological components of human milk. It may well be, however, that at least some of them may represent various pathogenic agents, including a hypothetical tumor agent.

The results obtained thus far do not exclude the possibility, therefore, that human milk samples may contain a tumor agent similar to that observed in certain strains of mice. It would then be necessary to assume that such a hypothetical tumor agent, or agents, may be quite common in perfectly healthy individuals and that they would not cause any harm or symptoms of disease in most instances. Such a possibility, however, is still purely speculative. Further biological, biochemical and immunological investigations are necessary to clarify the nature of the spherical particles which have been detected with the aid of the electron microscope in human milk samples.

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ATTEMPTS AT TUMOR VIRUS ISOLATION

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The discovery of a breast tumor-inducing factor in the milk of certain strains of mice by Bittner (1936), was followed by a number of studies on the properties of this factor, variously described as the milk agent, the mammary tumor-inducing agent, the mammary tumor inciter, or the Bittner virus. In this communication, this factor will be referred to as the milk agent or simply as the agent.

The properties of the agent in extracts of various tissues of high-cancer-strain mice were found to be similar to those of viruses. The agent remains active after lyophilisation (Bittner, 1941), treatment with glycerine (Bittner, 1942), desiccation (Dmochowski, 1944a), and after filtration through Seitz and Berkefeld filters of extracts of these tissues (Bittner, 1942). The agent in these extracts remains stable within a wide range of hydrogen ion concentrations from pH 5.0–10.2, and is not inactivated by petroleum ether and acetone (Barnum *et al.*, 1944), while heating of tissue extracts for one hour at 66°C. destroys its activity (Andervont and Bryan, 1944; Barnum *et al.*, 1944). Dialysis by stirring of Berkefeld filtrates of tumor tissue against distilled water, lasting for three hours, brings no diminution of the activity of the agent (Dmochowski and Stickland, 1949). When introduced into suitable susceptible mice, the agent was found to produce a high incidence of breast cancer which was maintained by successive generations of these mice (Andervont, 1945). In spite of the initial small dose, there was no sign of decrease in the activity of the agent, but it is still unknown whether the agent reproduces itself or the mice produce more of the agent. Although some preliminary studies on the behavior of the agent in the yolk sac of chick embryos have already been reported (Bittner, Evans and Green, 1945), a final conclusion about the multiplication of the agent must be based on further experiments along these lines. Gradual diminution of the agent in susceptible mice of some strains nursed for only a short period by their own mothers was reported by Bittner (1939a) and recently by Andervont (1949). While a small dose of the agent therefore may be sufficient to induce a number of tumors in susceptible mice, it may not be sufficient to maintain the incidence of tumors in the progeny of these mice. These observations need not necessarily mean that the milk agent differs from other known viruses. Even the long latent period of tumor development does not separate the agent from other viruses, in view of the now known long latent period of "scrapie" virus (Greig, 1940) in sheep. This is further strengthened by the recent discovery of the transmission of the virus of encephalomyelitis in the milk of mice (Magnus and Magnus, 1949) and of the possible transmission of the agent through the sperm of male mice (Andervont and Dunn, 1948; Foulds, 1949; Bittner, 1950; Dmochowski, 1951), similar to that of the "scrapie" virus and of the virus of fowl paralysis (Blackmore, 1934).

In view of the similarity of the properties of the agent to those of known viruses, a number of experiments was carried out in an attempt to gain further knowledge of the nature of the agent by means of ultracentrifugation of extracts of lactating mammary tissues of high-cancer-strain mice (Vischer, Green, Bittner, Ball, and Siedentopf, 1942) and of extracts of breast tumor tissue and milk (Bryan, Kahler, Shimkin, and Andervont, 1942; Kahler and Bryan, 1943; Kahler and Andervont, 1948). These experiments demonstrated that the agent is a protein of high molecular weight, but complete sedimentation of the tumor-inducing principle was not achieved even after centrifugation at $110,000 \times g$. for one hour (Bittner, 1945). In another series of experiments, on the basis of tumor incidences observed after serial dilution of different fractions obtained by differential centrifugation of extracts of tumors and lactating breast glands in saline-buffers, it was concluded that the agent is associated with large granules sedimented after five minutes at $23,000 \times g$. and with microsomes sedimented after 90 minutes at $23,000 \times g$. (Barnum, Ball, and Bittner, 1947; Barnum, Huseby, and Bittner, 1948; Barnum and Huseby, 1950; Huseby, Barnum, and Bittner, 1950). These microsome fractions of lactating mammary glands, in spite of their high tumor-inducing activity [$0.00023 \mu g$ N (Barnum and Huseby, 1950)] showed no difference in the electron microscope from similar fractions of mammary glands of agent-free mice (Huseby, Barnum, and Bittner, 1950). It is, therefore, possible that the microsome fractions were, for the most part, composed of normal tissue constituents. Attempts at isolation of the milk agent by coagulation of casein or hydrolysis of proteins of milk from high-cancer-strain mice by crystalline chymotrypsin or by decalcification and dialysis of this milk, followed by two cycles of differential high-speed centrifugation, each at $120,000 \times g$. for 30 minutes, revealed a considerable amount of tumor-inducing activity to be still present in the supernatant (Graff, Moore, Stanley, Randall, and Haagensen, 1949). The deposits sedimentable at this speed showed in the electron microscope spherical particles of 500–1500 Å diameter and of an average diameter of 980 Å. Similar particles could not be isolated from milk of low-cancer-strain mice. The fraction sedimentable from milk of high-cancer-strain mice showed two components in the optical centrifuge and in the Tiselius cell and both components were active. The purified preparations of high-cancer-strain milk were found to be active in dilutions containing $0.008 \mu g$ of nitrogen. It was, however, not determined in these experiments (Graff *et al.*, 1949) whether the tumor-inducing activity is associated exclusively with the sedimenting particles. Finally, chemical studies of the isolated microsome fractions of tumor and mammary gland tissue, carried out by Barnum and Huseby (1950), demonstrated that the activity of the agent remained unaltered, in spite of the removal of at least 95 per cent of pentose nucleic acid from these fractions.

In our attempts at separation of the milk agent, several extraction procedures were applied to various normal and malignant tissues and to milk of both high- and low-cancer-strain mice (Passey, Dmochowski, Astbury, and Reed, 1947; Passey, Dmochowski, Reed, and Astbury, 1950). Electron

microscopy offers a valuable help in the preliminary stages of the development of purification procedures for a given virus (Beard, 1945). Each step of the extraction procedure was therefore examined in the electron microscope and also tested biologically in suitable test mice. Although electron microscopy often permits no measurements of the purity of a given preparation of the agent, it served as a criterion of morphological homogeneity and supplied data on the size, shape, and structure of particles found in extracts of various mouse tissues prepared in different ways. A method of extraction of either fresh or frozen-dried tissues of both agent-carrying and agent-free mice was finally adopted. The method was as follows:

Desiccation of Material. Weighed amounts of tissues were either placed in Petri dishes or in ampoules and frozen in CO_2 . Petri dishes, which were used when the material was going to be examined on the following day, were placed in a desiccator attached by an inch-wide glass tubing to a "Speedivac" Model 2, two-stage rotary vacuum pump giving a vacuum of 0.00001 mm. Hg and having a displacement of 48 litres a minute (W. Edwards & Co., Ltd., London). Ampoules with frozen material, which were used when the material was intended for storage, were attached to the same one-inch-wide tubing by means of a circular arrangement which allowed for the simultaneous attachment of four ampoules. Between the desiccated material and the pump, a condenser of the same one-inch-wide glass tubing was built in, and placed in a "Thermos" flask with CO_2 ice to act as a moisture trap. The average desiccation time was 12 hours. After the desiccation, the ampoules were sealed off in such a way as to preserve the vacuum and were then stored in a solid CO_2 insulated box for several weeks or months until the time of use, when, as in the case of material desiccated in Petri dishes, the material was further treated in the following way:

Preparation of Extracts. Weighed amount of desiccated tissue was ground in ice-cooled mortar with sand and petroleum ether of boiling point below 40°C . which was gradually added to a final proportion of one ml. to ten mg. of dried tissue. The ether was then allowed to settle and was pipetted off. Extraction with petroleum ether of the same low-boiling point in Soxhlet apparatus lasting one hour was also used and found to give good results, as observed in the electron microscope. After treatment with petroleum ether, the residue was ground with distilled water which was gradually added to a final proportion of one ml. to ten mg. of the original dried material. The distilled water suspension was then centrifuged for 30 minutes at $2,500 \times g$., the resulting deposit discarded, and the supernatant filtered through Whatman No. 542 filter paper. Trypsin was then added to the filtrate in proportions according to the activity of trypsin, which was prepared by Dr. L. H. Stickland in the following way.

Five grams of powdered Allen & Hanbury trypsin were resuspended in 25 ml. of distilled water and dialysed against at least three changes of distilled water at 4°C . during the course of $2\frac{1}{2}$ –3 days. The dialysed suspension of trypsin was then centrifuged at $2,500 \times g$. for 15 minutes and the supernatant filtered through Whatman No. 1 filter paper. The filtrate in several dilutions (0.4 ml. of 1:20, 1:40, 1:80) was tested for its activity by clotting

of two ml. of "calcified milk" (ten ml. of N calcium chloride added to 50 ml. of fresh milk with water added to make up the volume to 100 ml.) at 40°C. Trypsin, which, in dilution of 0.4 ml. of 1:40, produced clotting of milk at an average time of 40 seconds (in three or more readings), was taken as an arbitrary unit of activity. If freshly prepared trypsin in dilution of 0.4 ml. of 1:40 produced clotting of milk at an average time 40 seconds, then 0.1 ml. of undiluted trypsin was added to 20 ml. of tissue extract. If clotting took place after 80 seconds, then trypsin was taken as half as strong and 0.2 ml. of undiluted trypsin was added to 20 ml. of tissue extract, and so on.

After addition of trypsin to the tissue extract, the pH was adjusted to between 7.4–7.6 and the extract incubated with trypsin for 30 minutes at 37°C. After incubation with trypsin, the extract was filtered through Berkefeld N candle and examined in the electron microscope by Dr. R. Reed of the Department of Biomolecular Structure, Leeds University. For electron microscopy, the Berkefeld filtrates of mouse tissue extracts were used in dilution of one part of extract to 500 parts of filtered distilled water. To achieve an even distribution of particles and to prevent their aggregation, the drops of diluted extract, after being placed on collodion mounting films, were immediately drained with filter paper to obtain thin layers of extract on the filmed grids. In this way, particle aggregation was minimized. After drying, the mounted grids were shadowed with gold or chromium.

As already reported, electron micrographs of extracts of normal, and malignant tissues, and of milk of mice of several high-cancer-strains, prepared by this method, revealed the presence of large numbers of spherical particles, mostly of an average diameter of 200–300 Å. Electron micrographs of similarly prepared extracts of normal and malignant tissues and of milk of several low-cancer strains have only occasionally shown particles of similar size: certainly fewer in number than those of high cancer strain tissue extracts (Passey, Dmochowski, Astbury, and Reed, 1947; Passey, Dmochowski, Astbury, Reed, and Johnson, 1948, 1950, 1951; Passey, Dmochowski, Reed, and Astbury, 1950).

The size distribution of the particles in extracts of mammary tumors from three different high-cancer strains was examined by Mr. G. Eaves and is shown on FIGURE 1. As can be seen, about 90 per cent of particles in extracts of RIII and A strain mammary tumors were found to be of 200–300 Å average diameter, while, in extracts of C3H strain mammary tumor, approximately 90 per cent of particles were counted to be between 150 and 250 Å average diameter. It is possible that particles of larger size than those normally encountered are, at least in many cases, aggregates and often symmetrical aggregates of smaller particles, as can be seen from FIGURE 2. It may be that various steps in the extraction procedure, such as desiccation, trypsinisation, treatment with petroleum ether, lead to the break up of larger particles. This may account for the difference in the size of particles observed by Graff *et al.* (1949) and that found by us.

In any assessment of the purity of a virus preparation, not only tests of its physical homogeneity by means of electron microscopy, ultracentrifugation, and electrophoresis must be carried out, but the biological activity of the

preparation also must be tested (Beard, 1945). In spite of the long latent period before any visible signs of the milk agent's activity develop, the biological tests must be carried out as with any virus preparation. Further, preparations of the milk agent showing high activity are of great importance for any final conclusions about the purity of the material. Serial dilutions of preparations of material containing the agent were tested by Barnum *et al.* (1947, 1948); Huseby *et al.* (1950); Barnum and Huseby (1950), and they served as a basis for the conclusion that the agent is present in mitochondrial and microsomal fractions of mammary gland and tumor tissue. There is no doubt that such tests give statistically significant results providing they are carried out on a sufficiently large number of test mice. The results of the method of serial dilutions of preparations of the milk agent, employed by Barnum *et al.* (1947, 1948) and Huseby *et al.* (1950), may

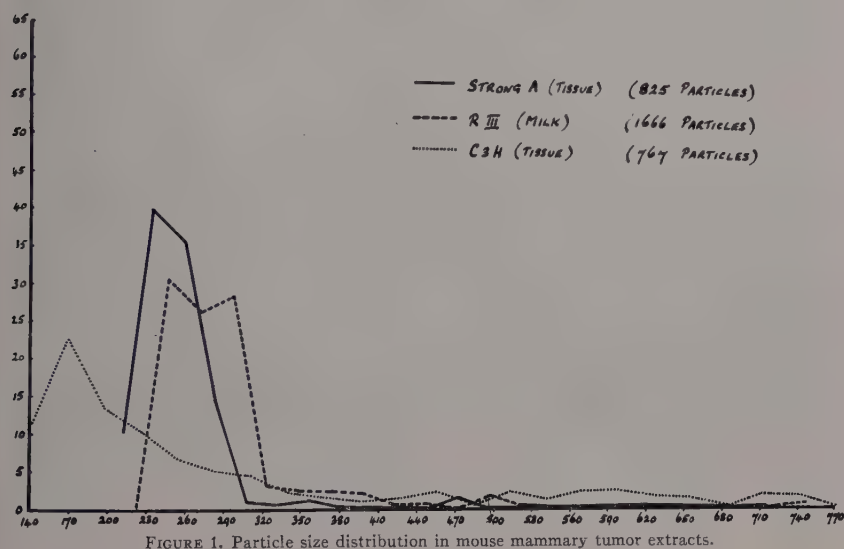


FIGURE 1. Particle size distribution in mouse mammary tumor extracts.

give rise, however, to certain doubts, based on the following observations. Some of the tested material gave approximately equal distribution of tumors throughout the whole series of dilutions used. Other material showed no activity at lower dilutions and high activity at higher dilutions, which however, was not maintained or increased in even higher dilutions. Still other material showed increased activity in higher dilutions, decrease of activity in still higher dilutions, followed by an increase in further dilutions. It is not known whether a sufficiently large number of test mice would give more constant results and it may well be that the variable results were produced by the different states of aggregation of the agent.

Biological tests of extracts of both high- and low-cancer-strain tissues in the present experiments were carried out with undiluted material injected in 0.5 ml. quantities either subcutaneously or intraperitoneally into (C57 × RIII) F₁ hybrid female mice which served as test mice. Extracts of both

high- and low-cancer-strain tissues were given, as far as possible, to litter mates which were subjected to forced breeding, that is, allowed to have three litters in quick succession and then bred in a normal way. In later experiments, the (C57 \times RIII) F₁ hybrids were not forcibly bred, but allowed to breed normally. The final results of biological tests are presented

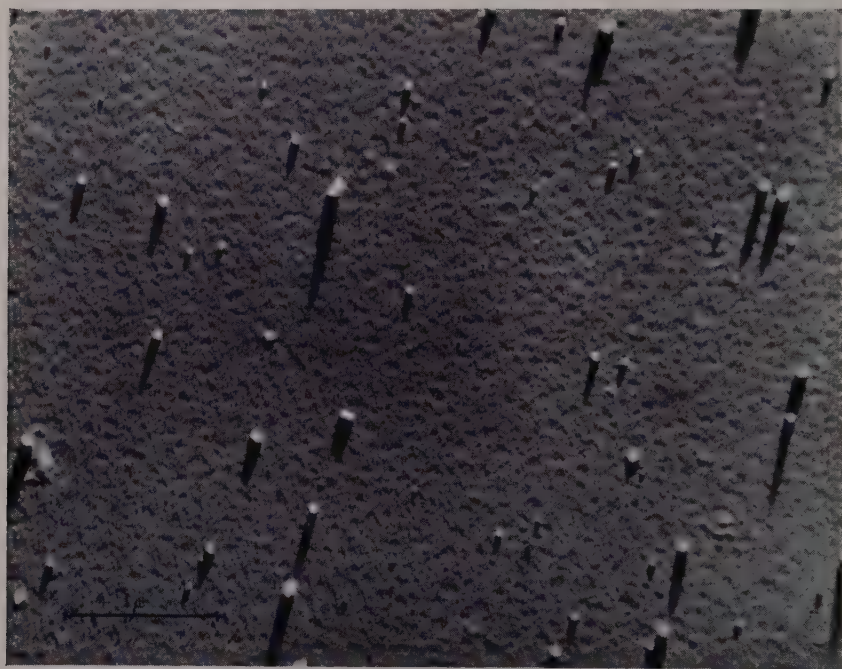


FIGURE 2. C3H (high cancer strain). Male breast tumor induced by painting with oestrone and methylcholanthrene; treated with petroleum ether, extracted with distilled water, treated with trypsin and filtered through a Berkefeld candle. The variation in particle size, due to aggregation, can be clearly seen.

TABLE 1

<i>Material with particles</i>	<i>Number of mice alive at the earliest tumor appearance</i>	<i>Number of tumors</i>	<i>Percentage of tumors</i>	<i>Average age of mice with tumors (months)</i>	<i>Average age of mice with- out tumors (months)</i>
Present.....	221	80	36.2	14.5	18.1
Absent.....	185	7	3.8	18.2	17.2

in TABLE 1, which is comprised altogether of 48 experiments with extracts of various tissues of different high- and low-cancer-strain mice. In 28 experiments, an average tumor incidence of 36 per cent was obtained in test mice injected with extracts of high-cancer-strain tissues which contained the characteristic particles, while, in another 20 experiments, an average tumor incidence of only three per cent was observed in test mice given extracts of

agent-free tissues which contained few or no particles. An analysis of the influence of forced breeding on the incidence of tumors in the test mice injected with both types of material is shown in TABLES 2 and 3. As can be seen from these tables, although forced breeding has influenced the tumor incidence in the test mice injected with low-cancer-strain tissue extracts it has not affected the tumor incidence in the test mice given extracts of high-cancer-strain tissues. A comparison of the tumor incidences in both forcibly and normally bred test mice, whether injected with material from high- or low-cancer-strain mice, shows that they differ significantly. This leads to the conclusion that mammary tumors observed in the test mice injected with extracts of various high-cancer-strain tissues containing the

TABLE 2

<i>Number of hybrids</i>	<i>Material with particles</i>	<i>Breeding</i>	<i>Number of tumors</i>	<i>Percentage of tumors</i>	<i>Average age of mice with tumors (months)</i>	<i>Average age of mice without tumors (months)</i>
94	Present	Forced	23	24.5	14.4	18.1
127	Present	Normal	57	44.8	14.6	18.0
100	Absent	Forced	6	6.0	15.5	17.6
85	Absent	Normal	1	1.2	22.0	16.6

TABLE 3

<i>Number of hybrids</i>	<i>Material with Particles</i>	<i>Breeding</i>	<i>Number of tumors</i>	<i>Percentage of tumors</i>	<i>Average age of mice with tumors (months)</i>	<i>Average age of mice without tumors (months)</i>
94	Present	Forced	23	24.5	14.4	18.1
100	Absent	Forced	6	6.0	15.5	17.6
127	Present	Normal	57	44.8	14.6	18.0
85	Absent	Normal	1	1.2	22.0	16.6

characteristic particles were produced by the milk agent and not by forced breeding or any other factor.

An analysis of the tumor incidence noted after different successive steps in the preparation of extracts of both high- and low-cancer-strain tissues is given in TABLES 4-7. It can be seen from these tables that the different treatments had a variable deleterious effect on the activity of the milk agent, providing the number of the test mice employed is accepted as sufficient to justify this conclusion. The possibility that a larger number of test mice would show a different effect of the extraction procedures on the activity of the agent must however be borne in mind. It can be seen in TABLES 4, 5, and 6 that a variable tumor incidence was induced in the test mice by products of the same extraction procedures applied to different samples of the same type of agent-containing tissues. This may be the result of variations in the

structure of tumors taken from the same strain, also of differences in the age of the tumor and the age of mice at which they appeared and, to an even greater extent, it may be the result of differences in the original amount of the agent present in these tumors. In TABLES 4-7 attention was drawn to the tumor incidences and not to tumor ages following the different treatments, in view of the observation by Barnum and Huseby (1950) that the latent period of mammary tumor development does not reflect the amount

TABLE 4
TUMOR INCIDENCE AFTER DIFFERENT TREATMENTS

Tissue	Treatment	Number of experiment						Total
		1	2	3	4	5	6	
C3H Milk, Dried	PE/DW/Tr./ Berk.	$\frac{1-13M}{6}$	$\frac{6-11M}{15}$	$\frac{2-15M}{6}$	$\frac{1-16M}{4}$	$\frac{2-20M}{2}$	—	$\frac{12-15M}{33}$
C3H Milk, Dried	PE/DW	—	—	—	$\frac{1-21M}{4}$	—	—	$\frac{1-21M}{4}$
C3H Milk, Dried	DW	—	—	—	—	—	$\frac{15-12M}{28}$	$\frac{15-12M}{28}$

Numerators are the numbers of mice which developed tumors. Denominators are numbers of mice which died without tumors. M = months of tumor age (average).

TABLE 5
TUMOR INCIDENCE AFTER DIFFERENT TREATMENTS

Tumor	Treatment	Number of experiment						Total
		1	2	3 and 4	5 and 6	7 and 8	9	
C3H, Dried	PE/DW/Tr./ Berk.	$\frac{4-14M}{4}$	$\frac{3-12M}{6}$	$\frac{4-16M}{11}$	$\frac{2-15M}{12}$	$\frac{15-12M}{19}$	$\frac{2-10M}{6}$	$\frac{30-13M}{58}$
C3H, Dried	PE/DW/Berk.	—	$\frac{2-14M}{4}$	—	$\frac{0-—M}{10}$	—	—	$\frac{2-14M}{14}$
C3H, Fresh	PE/DW/Tr./ Berk.	—	—	$\frac{2-17M}{16}$	$\frac{0-—M}{2}$	$\frac{3-12M}{10}$	—	$\frac{5-15M}{28}$
C3H, Fresh	PE/DW/Berk.	—	—	$\frac{0-—M}{2}$	$\frac{5-10M}{11}$	—	—	$\frac{5-10M}{13}$
C3H, Fresh	DW/Berk.	—	—	—	$\frac{8-9M}{11}$	$\frac{3-12M}{9}$	—	$\frac{11-11M}{20}$
C3H, Fresh	DW/Tr./Berk.	—	—	—	$\frac{1-9M}{6}$	—	—	$\frac{1-9M}{6}$

Numerators are numbers of mice which developed tumors. Denominators are numbers of mice which died without tumors. M = months of average tumor age.

of the agent accurately enough to be used in estimating the content of the agent in different preparations. The total tumor incidence after different treatment of some high-cancer-strain tissues is shown in TABLE 7.

The tumor incidence after treatment of some low-cancer-strain tissues is shown in TABLE 8. The tumor age with one exception was considerably higher than that of the test mice given extracts of high-cancer-strain tissues, in spite of the forced breeding, after which all except one tumor appeared in the test mice injected with low-cancer-strain tissue extracts. The ap-

TABLE 6
TUMOR INCIDENCE AFTER DIFFERENT TREATMENTS

Tumor	Treatment	Number of experiment				Total
		1 and 2	2 and 3	4	5	
RIII Dried	PE/DW/Tr./Berk.	$\frac{4-14M}{10}$	—	$\frac{3-12M}{12}$	$\frac{1-16M}{8}$	$\frac{8-14M}{30}$
RIII Dried	PE/DW/Berk.	—	$\frac{8-15M}{19}$	—	—	$\frac{8-15M}{19}$
RIII Dried	DW	—	—	$\frac{3-12M}{10}$	$\frac{3-13M}{9}$	$\frac{6-13M}{19}$
RIII Fresh	PE/DW/Berk.	$\frac{4-13M}{8}$	$\frac{2-13M}{9}$	—	—	$\frac{6-13M}{17}$
RIII Fresh	DW/Tr./Berk.	$\frac{1-12M}{11}$	—	—	—	$\frac{1-12M}{11}$
RIII Fresh	PE/DW/Tr./Berk.	$\frac{1-8M}{8}$	$\frac{2-10M}{10}$	—	—	$\frac{3-9M}{18}$

Numerators are numbers of mice which developed tumors. Denominators are numbers of mice which died without tumors. M = months of average tumor age.

TABLE 7
TOTAL TUMOR INCIDENCE AFTER DIFFERENT TREATMENTS

Treatment	Type of tissue				
	C3H Milk dried	RIII Tu-mor dried	RIII Tu-mor fresh	C3H Tu-mor dried	C3H Tu-mor fresh
D.W.	$\frac{15-12M}{28}$	$\frac{6-13M}{19}$	—	—	—
D.W./Berk.	—	—	—	—	$\frac{11-11M}{20}$
P.E./D.W.	$\frac{1-21M}{4}$	—	—	—	—
P.E./D.W./Berk.	—	$\frac{8-15M}{19}$	$\frac{6-13M}{17}$	$\frac{2-14M}{14}$	$\frac{5-10M}{13}$
D.W./Tr./Berk.	—	—	$\frac{1-12M}{11}$	—	$\frac{1-9M}{6}$
P.E./D.W./Tr./Berk.	$\frac{12-15M}{33}$	$\frac{8-14M}{30}$	$\frac{3-9M}{18}$	$\frac{30-13M}{58}$	$\frac{5-15M}{28}$

Numerators are numbers of mice which developed tumors. Denominators are numbers of mice which died without tumors. M = months of average tumor age.

pearance of tumors in these mice after forced breeding, as well as their late appearance, indicates that the origin of these tumors was the same as that of tumors occasionally observed in other hybrid mice used as test mice and reported by a number of workers (Bittner, 1939b, Gardner, 1941; Strong, 1943; Dmochowski, 1944b; Andervont, 1945; Foulds, 1947).

As soon as the difference between the presence of the characteristic particles in extracts of high- and low-cancer-strain tissues was observed, ultracentrifugation experiments were carried out in an attempt to ascertain the relationship of the tumor-inducing principle to the characteristic particles. Electron microscope examination, as well as the biological tests of deposits and supernatants, were carried out at the same time after each centrifugation. Both deposits and supernatants, in comparable dilutions, were injected in 0.5 ml. quantities intraperitoneally into (C57 \times A) F₁ hybrids. Trypsinised and non-trypsinised extracts of spontaneous mammary tumor tissue were centrifuged in the Beams-Pickels ultracentrifuge at speeds equivalent to 40,000–120,000 \times g. for periods varying from one to two hours. After centrifugation for two hours at 120,000 \times g., the supernatant fluid

TABLE 8
TUMOR INCIDENCE AFTER TREATMENT OF SOME LOW-BREAST-CANCER-STRAIN TISSUES

Tissue	Treatment	Number of experiment					Total
		1	2	3	4	5	
IF Strain breast tumor	PE/Dist.-W./Tr./Berk.	0—14M* $\frac{7}{7}$	0—19M* $\frac{6}{6}$	1—22M $\frac{6}{6}$	—	—	1—22M (17M)* $\frac{19}{19}$
C57 Strain breast tumor	PE/Dist.-W./Tr./Berk.	0—20M* $\frac{13}{13}$	0—21M* $\frac{7}{7}$	0—12M* $\frac{12}{12}$	—	—	0—18M* $\frac{32}{32}$
C57 Strain breast milk	PE/Dist.-W./Tr./Berk.	1—19M $\frac{18}{18}$	3—19M $\frac{18}{18}$	0—17M* $\frac{11}{11}$	—	—	4—19M (18M)* $\frac{47}{47}$
C57 Strain stomach milk	PE/Dist.-W./Tr./Berk.	0—14M* $\frac{13}{13}$	1—22M $\frac{18}{18}$	0—17M* $\frac{12}{12}$	0—15M* $\frac{6}{6}$	—	1—22M (15M)* $\frac{49}{49}$
C57 Strain stomach milk	Dist.-W.	—	—	—	0—15M* $\frac{6}{6}$	—	0—15M* $\frac{6}{6}$
C57 Strain spontaneous thymus tumor	PE/Dist.-W./Tr./Berk.	—	—	—	—	1—9M $\frac{6}{6}$	1—9M $\frac{6}{6}$

Numerators are numbers of mice which developed tumors. Denominators are numbers of mice which died without tumors. M = months of average tumor age.

* Average age of mice which died without tumors.

was found, in general, to be entirely free from particles when examined in the electron microscope (Passey, Dmochowski, Astbury, Reed and Johnson, 1948, 1950, 1951). When centrifugation at 120,000 \times g. lasted less than two hours, or, even more so, after centrifugation for one hour at 60,000 \times g. or 40,000 \times g., the supernatant fluids were found to contain large numbers of the characteristic particles. The deposits examined after each centrifugation were also found to contain the typical particles. A summary of the results of biological tests of the centrifuged material is given in TABLE 9. It can be seen that very little, if any, tumor-inducing activity was left in supernatants in which no particles could be seen in the electron microscope. A more detailed presentation of the results of several experiments is given in TABLE 10. From the results of the biological tests, it can be seen that the tumor-inducing activity of the deposits was of the same order as that of the

original extracts. Further, whenever particles were found in the supernatants, they also possessed tumor-inducing activity, although weaker than the original extracts or the deposits. On occasion, when the supernatants obtained by centrifugation at $120,000 \times g$. for two hours were found to contain the typical particles, possibly through contamination by less careful deceleration or pipetting off, these supernatants were also found to be active biologically. Only in one case, a supernatant obtained after centrifugation at $120,000 \times g$. for two hours of an extract of a fresh C3H strain mammary tumor tissue which had been extracted with distilled water and filtered

TABLE 9
ULTRACENTRIFUGATION AND ELECTRON MICROSCOPY

<div> <div>Number of mice with tumors</div> <div>Number of mice dying without tumors</div> </div>			
Original material	Sediment	Supernatant	Particles in supernatant
24/63 (38.1%)	29/65 (44.6%)	8/60 (13.3%)	Present
20/81 (24.7%)	28/85 (32.9%)	1/82 (1.2%)	Absent

TABLE 10
SUMMARY OF RESULTS
ULTRACENTRIFUGATION AND ELECTRON MICROSCOPY

Number of experiment	<div> <div>Number of mice with tumors</div> <div>Number of mice dying without tumors</div> </div>			
	Original material	Sediment	Supernatant	Particles in supernatant
41	14/30	20/46	5/43	Present
42	6/19	9/22	1/24	Absent
43	4/11	4/11	1/8	Present
44	6/22	5/8	2/9	Present
45	4/17	4/9	0/12	Absent
46	6/27	9/31	0/27	Absent
52	4/19	6/23	0/19	Absent

through Berkefeld candle, and in which no particles could be seen in the electron microscope, was found to be still active, giving one tumor in twelve of the surviving test mice.

Biological results after ultracentrifugation of extracts of mammary tumors of two high-cancer-strains, which had been prepared in various ways, are shown in TABLES 11 and 12.

The influence of different methods of treatment of C3H strain breast tumor tissues is shown in TABLE 11. While in the column of Experiment No. 1 the same fresh C3H strain tumor tissue was treated in different ways, the dried C3H breast tumor tissue was of different origin, and the biological results with this tissue therefore cannot be compared directly with those

obtained with the fresh tumor tissue. Comparison of the tumor incidence obtained with different extracts of C3H fresh tumor tissue in the first experiment with that induced by similar extracts of another sample of fresh C3H tumor in the second experiment, indicates individual variation in the response of various samples of tissue of the same origin to different methods

TABLE 11
ULTRACENTRIFUGATION OF HIGH-CANCER-STRAIN TUMORS

Tumor tissue	Type of treatment	Experiment No. 1			Experiment No. 2		
		Original extract	Deposit	Super-natant	Original extract	Deposit.	Super-natant
C3H, Fresh	Dist.-W./Berk.	$\frac{8-9M}{11}$	$\frac{6-10M}{12}$	$\frac{1-15M}{12}$	$\frac{3-12M}{9}$	$\frac{3-12M}{9}$	$\frac{1-14M}{12}$
	Dist.-W./Tryps./Berk.	$\frac{1-9M}{6}$	$\frac{8-12M}{12}$	$\frac{3-8M}{11}$	—	—	—
	Dist.-W./Petr.-Eth./Berk.	$\frac{5-10M}{11}$	$\frac{3-11M}{11}$	$\frac{0-M}{12}$	—	—	—
	Dist.-W./Petr.-Eth./Tryps./Berk.	$\frac{0-M}{2}$	$\frac{3-11M}{11}$	$\frac{1-18M}{8}$	$\frac{3-12M}{10}$	$\frac{6-15M}{13}$	$\frac{0-M}{12}$
C3H, Dried	Dist.-W./Petr.-Eth./Tryps./Berk.	$\frac{4-16M}{11}$	$\frac{4-13M}{11}$	$\frac{1-10M}{8}$	—	—	—

Numerators are numbers of mice which developed tumors. Denominators are numbers of mice which died without tumors. M = months of average tumor age.

TABLE 12
ULTRACENTRIFUGATION OF HIGH-CANCER-STRAIN TUMORS

Tumor tissue	Type of treatment	Experiment No. 1			Experiment No. 2		
		Original extract	Deposit	Super-natant	Original extract	Deposit	Super-natant
RIII, Fresh	Petr.-Eth./Dist.-W./Berk.	$\frac{4-13M}{9}$	$\frac{4-15M}{11}$	$\frac{0-M}{10}$	$\frac{2-13M}{9}$	$\frac{2-12M}{9}$	$\frac{0-M}{10}$
	Dist.-W./Tryps./Berk.	$\frac{1-12M}{11}$	$\frac{4-15M}{12}$	$\frac{0-M}{8}$	—	—	—
	Petr.-Eth./Dist.-W./Tryps./Berk.	$\frac{1-10M}{8}$	$\frac{1-13M}{8}$	$\frac{0-M}{8}$	$\frac{2-10M}{10}$	$\frac{4-13M}{14}$	$\frac{0-M}{9}$
RIII, Dried	Dist.-W.	$\frac{3-12M}{10}$	—	—	$\frac{3-13M}{9}$	—	—
	Petr.-Eth./Dist.-W./Tryps./Berk.	$\frac{3-12M}{12}$	$\frac{5-12M}{18}$	$\frac{2-16M}{9}$	$\frac{1-16M}{8}$	$\frac{4-11M}{9}$	$\frac{0-M}{12}$

Numerators are numbers of mice which developed tumors. Denominators are numbers of mice which died without tumors. M = months of average tumor age.

of extraction. The activity of the deposits compares favorably with the activity shown by the original starting material. The tumor-inducing property of the supernatants was found to be parallel with the presence of typical particles in these supernatants. In Experiment No. 1, the extracts of fresh tumor tissue were centrifuged at $113,000 \times g$. for one hour, and in Experiment No. 2 they were centrifuged for two hours at $120,000 \times g$., like the extracts of dried tissue in Experiment No. 1. In each case, when the super-

natants contained the particles, they were also found to be biologically active. It is obvious that more experiments on a greater number of mice are required to reach a definite conclusion how the different extraction procedures affect the activity of the agent present in various mammary tumors of the same strain.

Again in TABLE 12, the results obtained with dried RIII tumor tissues in both experiments cannot be compared directly with those obtained with fresh tumor tissues because of their different origin, but they show again the individual variation in the response to different treatment of various samples of tumor tissue of the same strain. In only one case the supernatant showed tumor-inducing activity and a number of particles was observed in this supernatant, possibly as result of contamination, because the extract had been spun at $120,000 \times g$. for two hours. In all the other supernatants, no particles were seen in the electron microscope and they showed no activity.

The results of similar centrifugation procedures applied to extracts of breast tumors of three agent-free low-cancer strains are presented in TABLE

TABLE 13
ULTRACENTRIFUGATION OF LOW-CANCER-STRAIN TUMORS

<i>Tumor tissue</i>	<i>Type of Treatment</i>	<i>Original extract</i>	<i>Deposit</i>	<i>Supernatant</i>
C57 Strain Dried	Petr.-Eth./Dist.-W./ Tryps./Berk.	$\frac{0}{11} - 15M^*$	$\frac{0}{11} - 16M$	$\frac{0}{12} - 15M$
P Strain Dried	Petr. Eth./Dist.-W./ Tryps./Berk.	$\frac{0}{11} - 15M$	$\frac{0}{11} - 16M$	$\frac{0}{9} - 19M$
Y Strain Dried	Petr. Eth./Dist.-W./ Tryps./Berk.	$\frac{0}{6} - 15M$	$\frac{0}{12} - 15M$	$\frac{0}{10} - 15M$

M = months of average tumor age.

* Average age of mice which died without tumors.

13. As can be seen, no tumors were induced in the test mice injected with either the original material or the deposits obtained by centrifugation of these extracts at $120,000 \times g$. for two hours. Electron microscopy of these deposits revealed a varying number of particles, similar in size to those present in the deposits of the high-cancer-strain tumors, but in considerably smaller numbers.

A comparison of the number and size of particles observed in the deposits of two high- and two low-cancer-strain tumors is shown in FIGURES 3a, b, c, d. In the case of the high-cancer-strain tumors, areas are shown in the electron micrographs representing the average number of particles encountered in the deposits of these tumors as seen in an average number of 20 photographs. In the case of low-cancer-strain tumors, areas are shown representing the maximum number of particles encountered in similarly obtained deposits after ultracentrifugation of extracts of two low-cancer-strain tumors prepared in the same way as the extracts of high-cancer-strain tumors. Conclusions regarding any tissue extract, reported by Drs. W. T. Astbury and R. Reed, were based on the consideration of at least 15-20 photographs of each specimen examined. In the case of low-cancer-strain

tissue extracts, examination of photographs very often showed only occasional particles in each or only in some of the photographs and, in a few, the number shown in FIGURE 3 was observed. A comparison of the appearance of particles in extracts of dried tumor tissues of one low- and three high-cancer strains, all treated in the same way, is given in FIGURES 4a, b, c, d. The appearance of particles in extracts of two high-cancer-strain tumor

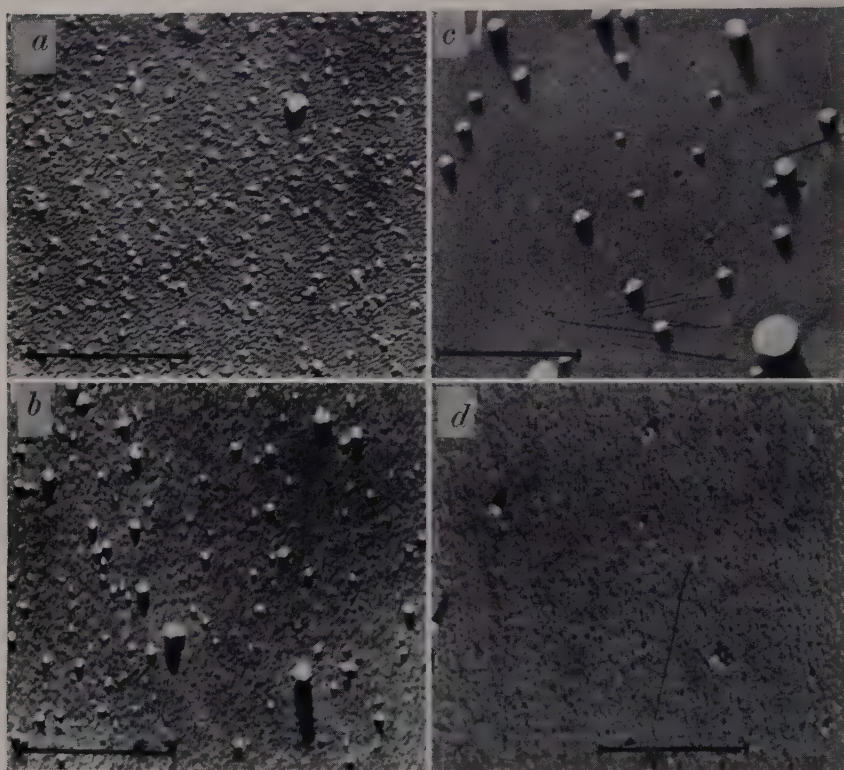


FIGURE 3a. RIII high-cancer-strain spontaneous mammary tumor, fresh tissue, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle. Deposit after centrifugation for two hours at $120,000 \times g$.

FIGURE 3b. C3H high-cancer-strain spontaneous mammary tumor, fresh tissue, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle. Deposit after centrifugation for two hours at $120,000 \times g$.

FIGURE 3c. C57 low-cancer-strain spontaneous mammary tumor, desiccated tissue, treated in the same way as the high-cancer-strain tissues. Deposit after centrifugation for two hours at $120,000 \times g$.

FIGURE 3d. Y low-cancer-strain spontaneous mammary tumor, desiccated tissue, treated in the same way as the high-cancer-strain tissue. Deposit after centrifugation after two hours at $120,000 \times g$.

tissues, before and after treatment with trypsin, can be seen in FIGURES 5a, b, c, d, which gives a comparison of the appearance of particles in each of the two tumor extracts when trypsinised and nontrypsinised.

Preliminary results of titration experiments, not yet completed, show that fractions of tumor extracts, treated with trypsin, with particles of the characteristic size and with a protein nitrogen content of $0.0008 \mu g.$, possess tumor-inducing activity.

Attempts at purification of the milk agent by chromatography on alumina demonstrated that whereas the starting material of a C3H strain mammary tumor, which had been desiccated, treated with petroleum ether, extracted with distilled water and filtered through Berkefeld N candle, gave a good yield of tumors at a dilution containing 0.03 $\mu\text{g.}$ of nitrogen per dose, one

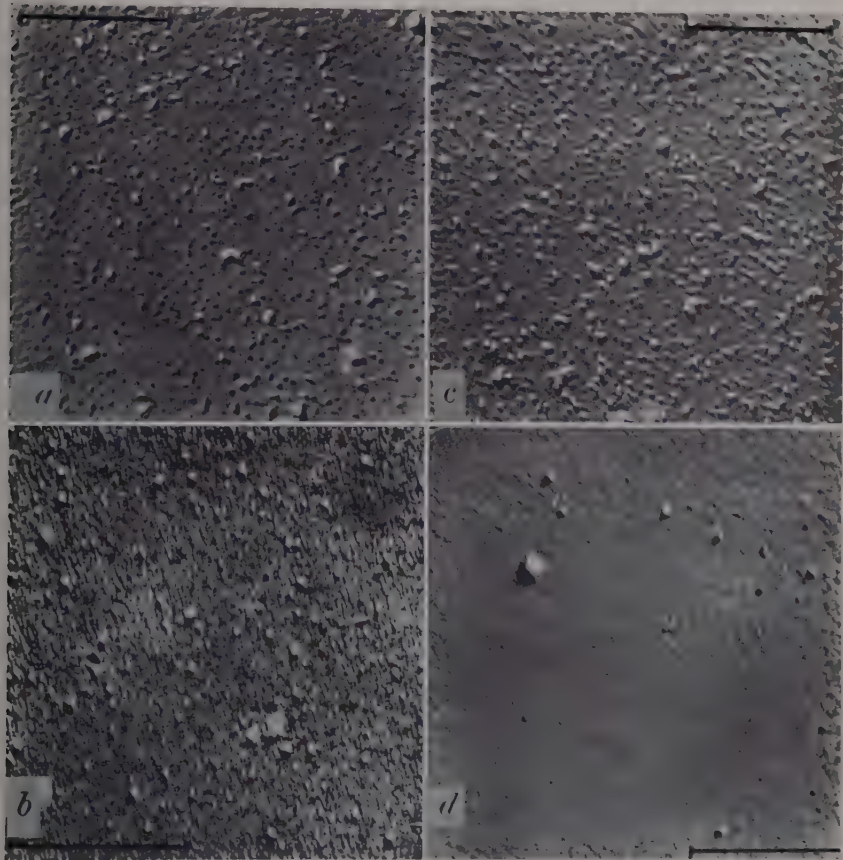


FIGURE 4a. RIII high-cancer-strain spontaneous mammary tumor, desiccated tissue, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

FIGURE 4b. C3H high cancer-strain spontaneous mammary tumor, desiccated tissue, treated in the same way.

FIGURE 4c. Strong A high-cancer-strain spontaneous mammary tumor, desiccated tissue, treated in the same way.

FIGURE 4d. C57 low cancer strain methylcholanthrene induced mammary tumor, desiccated tissue, treated in the same way.

of the fractions was still active in a dose containing 0.004 $\mu\text{g.}$ of nitrogen (Dmochowski and Stickland, 1950), although the activity of the original material in a comparable dose was not ascertained.

From the electron microscope examination of extracts of various high-cancer-strain tissues and the final assessment of the biological tests of these extracts, and further from the results of the centrifugation experiments with

extracts of high-cancer-strain tumors as shown in the electron microscope and in the biological tests, it is concluded that the tumor-inducing activity is associated with the typical particles.

While Graff *et al.* (1949) reported the results of biological tests of the deposits after centrifugation of treated RIII strain milk at $120,000 \times g$.

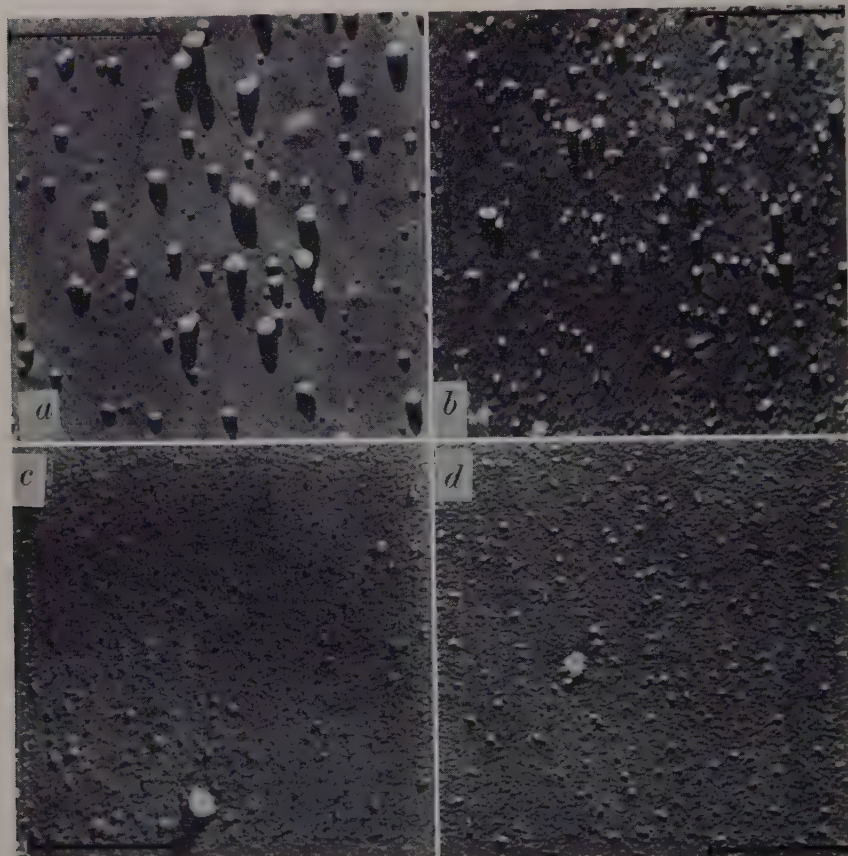


FIGURE 5a. C3H high-cancer-strain spontaneous tumor, fresh tissue, extracted with distilled water, filtered through Berkefeld N candle.

FIGURE 5b. Same C3H tumor tissue, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

FIGURE 5c. RIII high-cancer-strain spontaneous tumor, fresh tissue, treated with petroleum ether, extracted with distilled water, filtered through Berkefeld N candle.

FIGURE 5d. Same RIII tumor tissue, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

for 30 minutes, biological tests of the corresponding supernatants were not recorded. No reference was made either to electron microscope examination of the supernatants after this centrifugation. Graff *et al.* (1949), however, described the results of biological tests of supernatants and deposits after centrifugation of untreated RIII strain milk at $60,000 \times g$ for half-an-hour, in which no difference was found in the tumor-inducing activity between the supernatants and the deposits. They stressed the point that in their experi-

ments the association of the activity with the particles observed still remained to be determined. From the electron microscope examination of the different fractions obtained by various centrifugation procedures, previously reported (Passey, Dmochowski, Astbury, Reed, and Johnson, 1948, 1950, 1951), and the final results of the biological tests, now described, it is possible to conclude that the activity of the agent is associated with spherical particles mostly of 200–300 Å in diameter. The difference in results obtained by Graff *et al.* and our own may well be the result of the different treatment applied. Of further interest is the difference in the results with trypsinisation, which destroyed the tumor-inducing activity in the experience of Graff *et al.*, contrary to the observations recorded in the present experiments. It is possible that the preparation of trypsin employed, and further, its concentration and also the length of treatment with trypsin in the respective experiments, was responsible for the different observations on the influence of trypsin on the activity of the agent. As to the presence of spherical particles encountered in varying numbers in extracts of some low-cancer-strain tissues, and more frequently in the deposits after centrifugation of some low-cancer-strain mammary tumors, it may well be that this great range of characteristic particles seen in extracts of treated tissues of mice points to a certain critical threshold in the number of the particles required for the appearance of mammary tumor. A further possibility to be taken into consideration is a probable difference in the character of particles encountered in high- and low-cancer-strain tissues, those observed in high-cancer-strain tissue extracts after the treatment being intimately associated with the tumor-inducing activity, and those seen in similarly treated low-cancer-strain tissue extracts being the remaining normal tissue constituents.

It is realized that more elaborate and detailed infectivity studies will have to be carried out, now that good products have been obtained by means of ultracentrifugation, as shown in the electron microscope. Titration studies have already been applied, although it must be pointed out that they can only give a broad approximation of the tumor-inducing activity, and they may probably present difficulties in the final assessment of results similar to those encountered in titration experiments by Barnum *et al.* (1947, 1948) and Huseby *et al.* (1950). As a further step in the assessment of purity of the preparations, an electrophoresis experiment, in collaboration with Dr. D. H. Moore, was carried out with a concentrated preparation from C3H tumor tissue, subjected to 4.5 volts/cm. for eight hours in the Tiselius cell. The appearance of samples from the anode side is shown in FIGURES 6 and 7. These, as well as other samples, have been tested biologically, and although it is too early to draw final conclusions, it can already be reported that the sample illustrated in FIGURE 7 has already induced tumors in doses containing 10^8 particles, which doses are, of course, of a low order of infectivity.

In connection with the results obtained with mouse tissues, similar extraction procedures were applied to human breast tumors and milks of apparently healthy women and milks of women suffering from breast cancer. Electron microscope studies of these extracts revealed greatly varying numbers of spherical particles in nearly all extracts, although slightly larger

than those present in mouse tissues. The results can be seen in TABLE 14. Although the number of specimens, thirty-eight in all, is too small to allow any final conclusions, it can be stated that there appear to be more particles present in breast tumors and milks from women with breast cancer than in the milks from apparently healthy women (Passey, Dmochowski, Astbury, Reed, and Eaves, 1951). Electron micrographs of human breast tumors are shown in FIGURES 8 and 9, which illustrate the varying number of

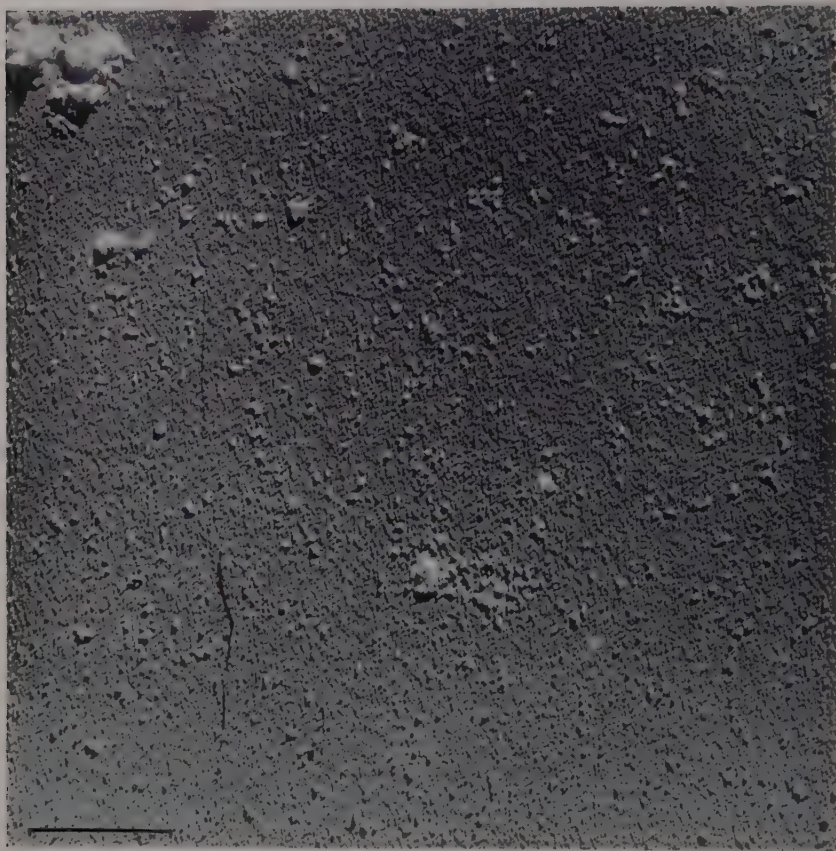


FIGURE 6. C3H mammary tumor tissue, extracted in phosphate buffer at pH 7.5, trypsinised. Sediment after centrifugation for two hours at $120,000 \times g$, ten times concentrated. Appearance of lower sample from the anode side in the Tiselius cell.

spherical particles observed in these tumors. Similar observations recorded with the milks of women with breast cancer are seen in FIGURES 10, 11 and 12, while those with the milks of apparently healthy women are shown in FIGURES 13 and 14.

The number of particles present in extracts of human breast cancer was found to be related to the histological appearance of tumors, those of the scirrhus type yielding fewer particles than other types. It is not known, at present, what connection, if any, these particles may have with the appear-

ance of breast cancer in women. Similar observations in milks of women whose relatives had breast cancer and also in some milks of apparently healthy women were reported by Gross, Gessler, and McCarty (1950). The particles described by these workers are of a larger size than those recorded in the present experiments, which may be the result of the different treatments applied in their extraction procedures. There is no doubt that these studies require continuation and extension and may perhaps help in the

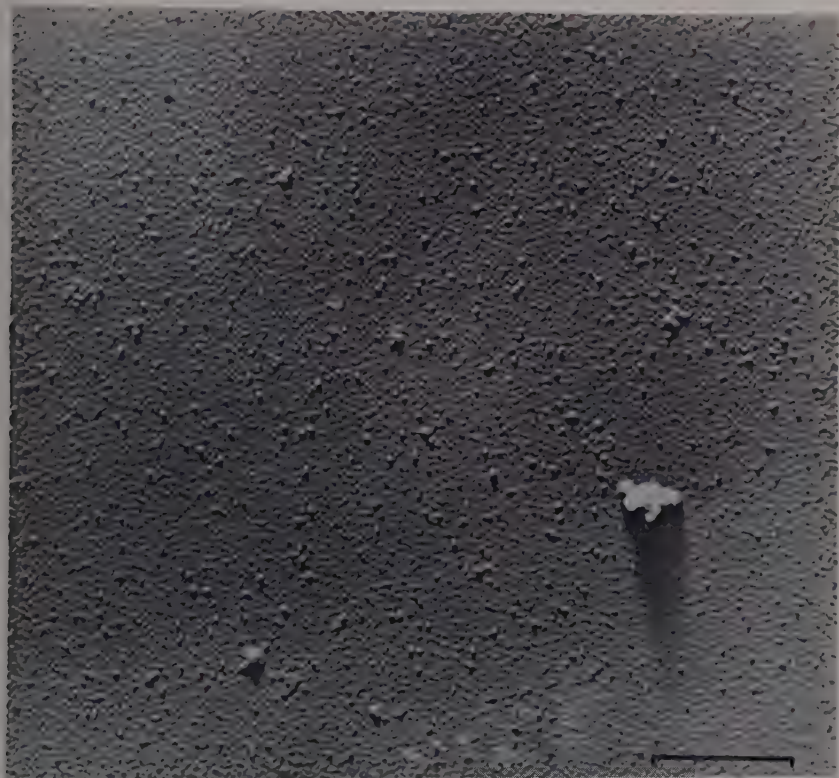


FIGURE 7. C3H mammary tumor tissue, extracted in phosphate buffer at pH 7.5, trypsinized. Sediment after centrifugation for two hours at 120,000 \times g, ten times concentrated. Appearance of upper sample from the anode side in the Tiselius cell.

evaluation of whether preventative measures, if any, are possible in the breast cancer of women.

In an attempt to find a possible relationship between the particles seen in extracts of mouse and human tumors and also in order to ascertain their serological properties, immunological studies were carried out on both types of particles.

Studies on the antigenic properties of the milk agent in preparations of different tissues were reported by a number of investigators and carried out by means of neutralization, complement fixation, and precipitation tests with rabbit, rat, and guinea pig sera.

Andervont and Bryan (1944) described neutralization of the agent in partially purified material obtained by ultracentrifugation of extracts of mammary tumors by sera induced in rabbits with similarly prepared extracts of mouse tumors. Normal rabbit sera and sera of rabbits bearing a transplantable rabbit carcinoma gave a similar, but much less marked, effect. They concluded that the milk agent is antigenic, but there were no controls with immune sera against mouse tissues, both normal and malignant, without the agent. Andervont and Bryan (1944) also found that the rabbit immune sera, injected into susceptible mice before they were given material

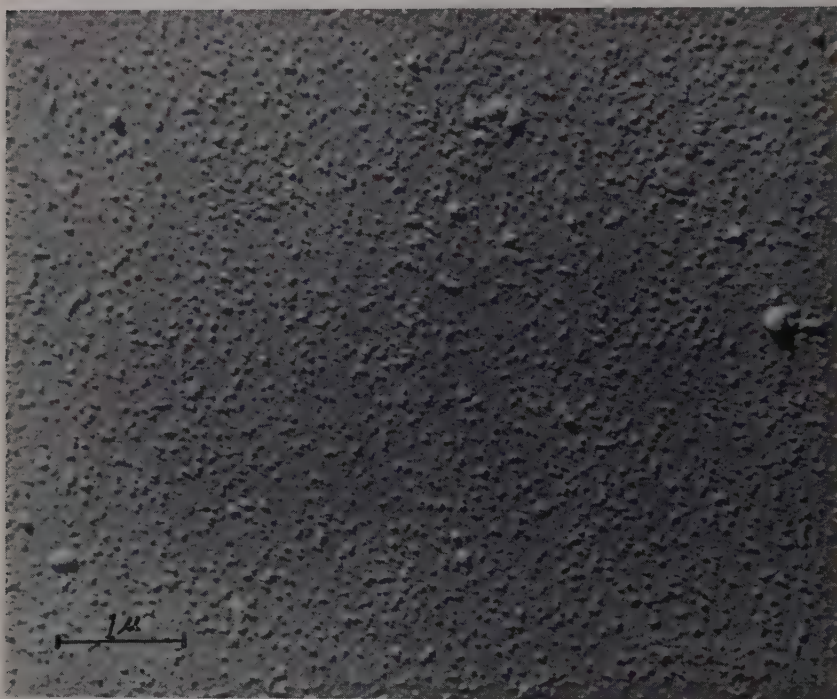


FIGURE 8. Human breast tumor, desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

containing the agent, prevented the development of tumors in these mice. Similar sera, although injected at an early age, failed to prevent the appearance of tumors in mice which obtained the agent during nursing (Bittner, 1948). Green, Moosey, and Bittner (1946) and also Green and Bittner (1946) similarly showed neutralization of the agent in ultracentrifugal deposits of breast tumor extracts by rabbit and rat immune sera prepared with this material, while antisera against material from normal mouse tissues failed to have a similar effect. Normal rabbit and rat sera showed a delaying and even an inhibiting effect, to a certain extent, on the agent present in this material. It is not known whether the normal tissues were from the same strain of mice from which the tumor material was obtained, but free

of the milk agent, or from another agent-free strain. It is difficult, therefore, to decide whether the neutralizing effect of sera was due to antibodies to the agent or to the tumor tissue.

Inhibition of the growth of mammary tumor cells by rabbit and rat immune sera induced by material from mammary tumor cells was demonstrated by Green (1946), while antisera against agent-free normal lactating mammary tissue and even normal rabbit sera delayed and even partially inhibited the growth of mammary tumor cells. Again, it was not ascer-

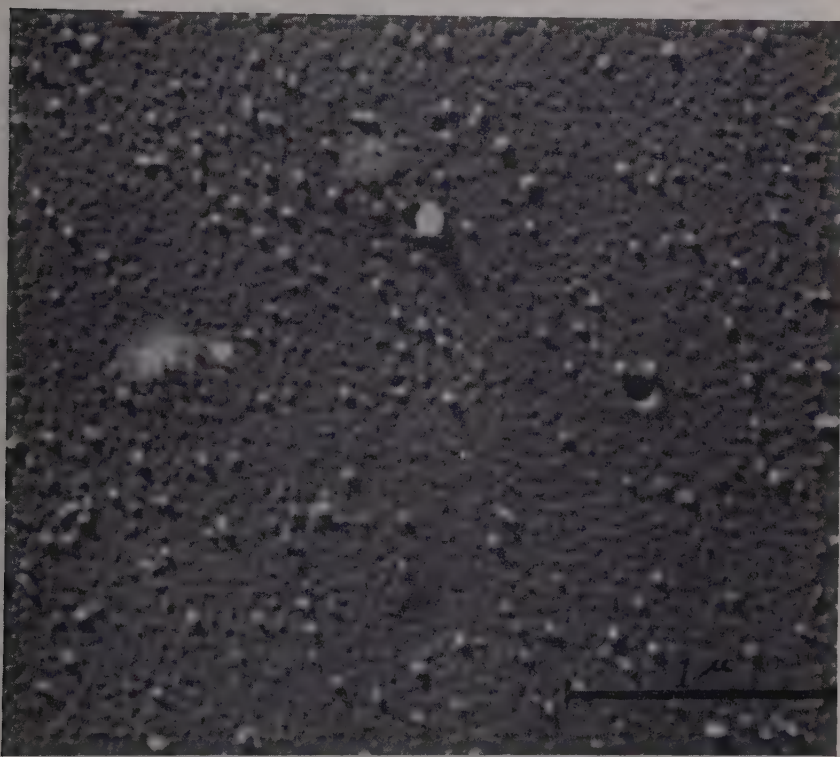


FIGURE 9. Human breast tumor, desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

tained what effect antisera against similarly prepared material from mammary tumors without the agent, preferably from genetically identical mice, would have exhibited and it is not clear therefore whether this growth-inhibitory effect was due to the agent itself or to antibodies against tumor tissue. Imagawa, Bittner, and Syverton (1950) observed a similar growth-inhibitory effect exerted by guinea pig immune sera against agent-containing spontaneous and transplantable mammary tumor tissue, while sera against normal mammary tissue had only a slight growth-delaying effect. They also found that sera of agent-carrying mice or mice bearing transplantable breast tumors with the agent had no effect at all. Further,

Imagawa, Syverton, and Bittner (1951) reported a similar growth-inhibitory effect of guinea pig immune sera induced by tumor material of one high-cancer strain on tumor cells of another high-cancer strain, while normal guinea-pig sera had no effect on tumor cells of either of these two strains. It would have been of interest to examine the effect of guinea pig immune sera induced by agent-free tumor material from other strains as well as genetically identical but agent-free strains. It is difficult to assess the discrepancy between these findings and the observations of Law (1949) who



FIGURE 10. Human cancer milk, desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

found that rabbit sera against normal lactating mammary tissues, both with and without the agent, inhibited the growth of high-cancer-strain tumors while antisera against spontaneous breast tumor tissue failed to show a similar effect. Gorer and Law (1949) reported no neutralizing antibodies to the milk agent in sera either of high-cancer-strain or hyperimmunised low-cancer-strain mice.

Imagawa, Green, and Halvorson (1948) demonstrated that rabbit sera against normal and malignant tissues of mice with the agent gave a high titre in precipitation tests with normal and malignant tissues of agent-carrying mice and only a low titre with normal tissues of agent-free mice.

Sera against normal tissues without the agent reacted only to a low titre with normal tissues of agent-free mice. They concluded that there is a common antigen in normal and malignant tissues with the agent which is absent from breast tissues without the agent, but controls with mammary tumor tissue without the agent were not reported. Law and Malmgren (1951) have recently shown that rabbit sera against material from normal and malignant breast tissues of mice genetically similar, with and without

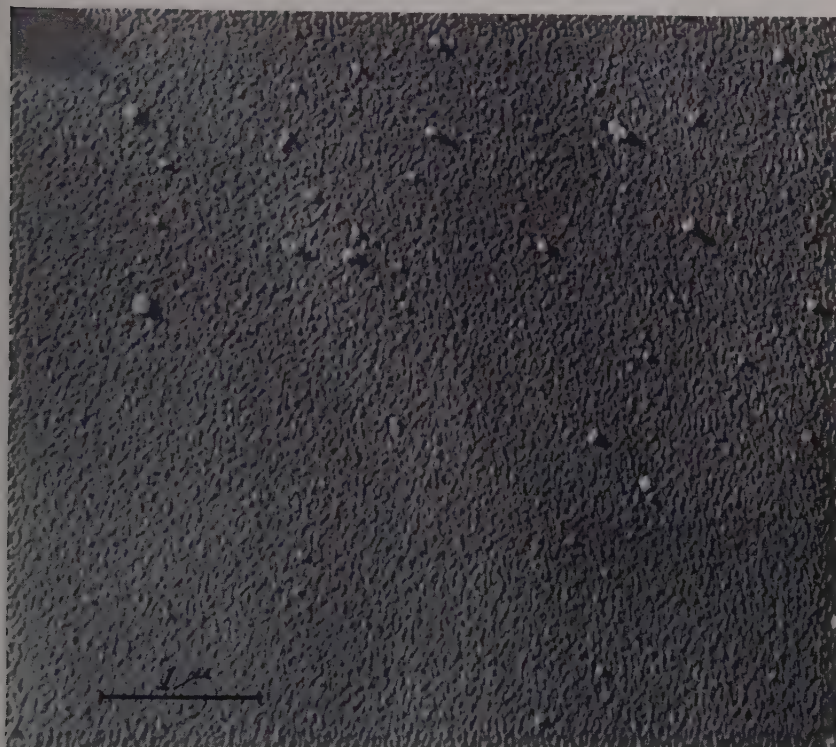


FIGURE 11. Milk from a woman with cancer of the breast. Milk desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld candle. Chromium shadowed.

the agent, reveal no difference in their growth-inhibitory effect on tumor cells.

Bennison (1948) demonstrated that extracts of spleen from agent-free mice gave a lower titre in complement fixation tests with rabbit sera against normal mammary tissue of agent-carrying mice than extracts of spleen from mice harboring the agent. Investigation of the antigenic properties of mitochondria from normal and malignant mammary tissues of mice, carried out by Malmgren and Bennison (1950), showed that antisera against ultra-centrifugal deposits of mammary tumor extracts or against mitochondria from mammary tumors reacted equally well in complement fixation tests with mitochondria from normal tissues of mice with and without the agent.

Similarly microsome fractions from tumors and spleens of genetically similar, both agent-carrying and agent-free mice, reacted equally well with rabbit antisera to microsome fractions from tumors of either types of mice (Malmgren *et al.*, 1951).

Thus, in some of the experiments so far recorded, the specificity of the antibodies induced with agent-containing material, whether examined in neutralization, complement fixation, or precipitation tests, was not entirely elucidated and in other experiments the lack of qualitative differences

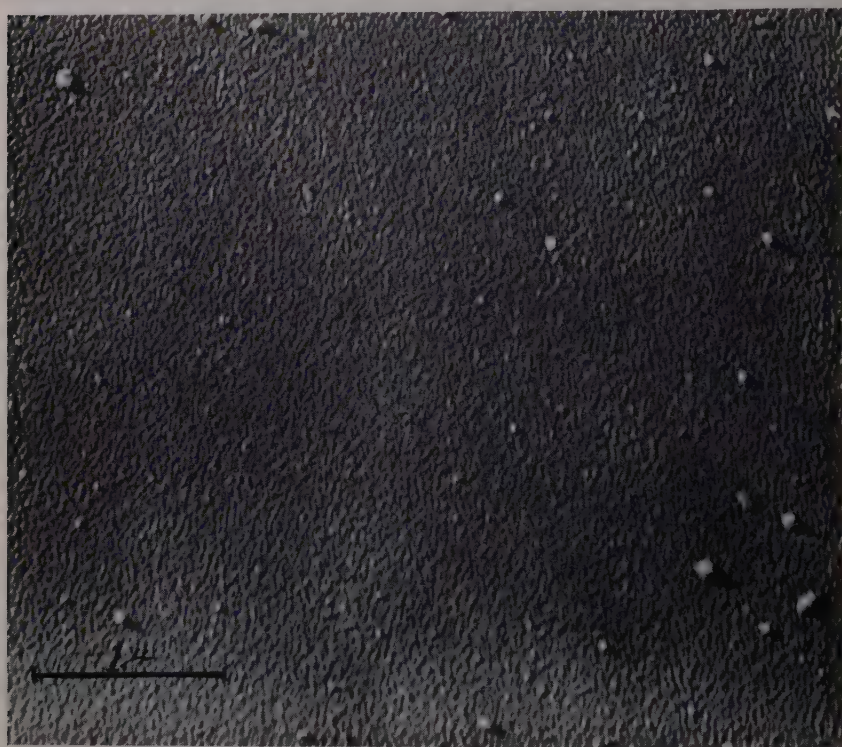


FIGURE 12. Human cancer milk, desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

stressed between the immune reactions induced by tissues from agent-free and agent-carrying mice.

In the present experiments, now in press, carried out in collaboration with Dr. L. Hoyle, complement fixation reaction was used because of its sensitivity and its successful use in the detection of antibodies to viruses of the same size as the typical particles present in extracts of mouse and human tissues. The technique of the test was similar to that described in previous experiments (Dmochowski, 1948). The method of preparation of antigens, both mouse and human, for immunization was similar to that which revealed the presence of the characteristic particles and their intimate association

with the tumor-inducing property. An investigation was carried out of sera of mice for the presence of antibodies against the agent and of the antigenic properties of the characteristic particles in ultracentrifugal deposits of mouse and human breast tumors. The mice were immunized by a total of three intraperitoneal injections, each of 0.5 ml. of the antigen from RIII strain tumor tissue, given at weekly intervals, and bled fourteen days after the last injection and their sera heated at 56°C. for 30 minutes. Each of the two series of four rabbits, used for immunization with mouse and human

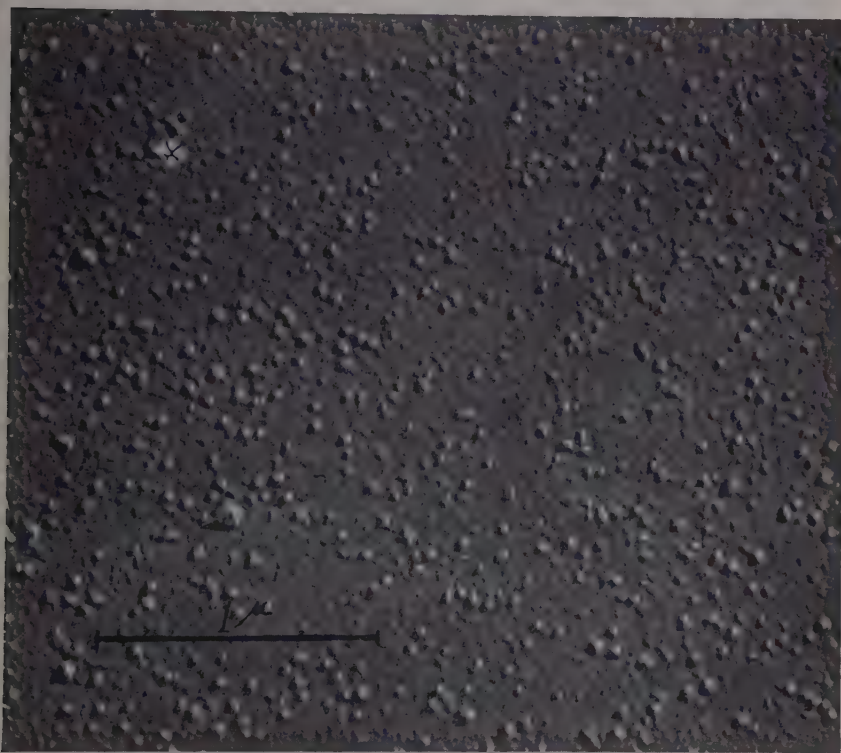


FIGURE 13. Normal human milk, desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld N candle.

breast tumor antigens, received two courses of intravenous injections, separated by a ten-day interval, and each of the courses comprised four injections given every other day. During the first course, the rabbits received one ml. of the respective antigens at each of the first two intravenous injections, which were followed by two ml. of each of the antigens given at all the following injections. The bleeding of rabbits followed 12 days after the last injection and a similar heat treatment was applied to that of the mouse sera.

Sera of the different strains of mice and their description as well as the various antigens employed in testing the sera are shown in TABLE 15. As

can be seen, no antibody could be detected in sera of high- and low-cancer-strain mice, whether normal, bearing different transplantable mammary

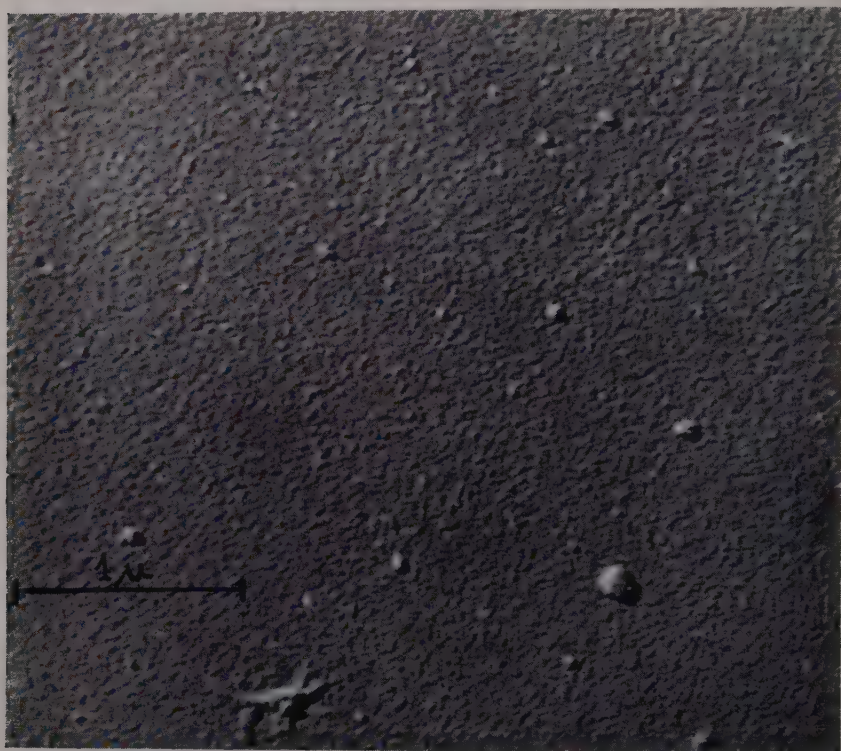


FIGURE 14. Milk from a normal woman. Desiccated, treated with petroleum ether, extracted with distilled water, treated with trypsin, filtered through Berkefeld candle. Chromium shadowed.

TABLE 14
HUMAN MATERIAL

<i>Number of particles</i>	<i>Breast tumor</i>	<i>Cancer milk</i>	<i>Normal milk</i>
++	8	4	1
+	3	2	4
±	6	2	6
—	1	0	1
Total.....	18	8	12

++ Numerous particles.
+ Less numerous particles.
± Only occasional particles.
— Particles absent.

tumors, or immunized with material containing the agent. The results suggest that the milk agent is not antigenic in mice and agree with those reported by Gorer and Law (1949) and by Imagawa, Bittner, and Syverton (1951) on the lack of neutralizing and cytotoxic antibodies in sera of mice.

TABLE 15

<i>Sera of mice</i>			<i>Antigens</i>	
<i>Milk agent</i>	<i>Strain</i>	<i>Description</i>	<i>Spontaneous tumor</i>	<i>Method of preparation</i>
Present	C3H	Normal Bearing C3H transplantable breast tumor (M.A. present) Immunized with RIII milk agent	RIII	1. 2% saline extract 2. Petroleum ether, distilled water, trypsin, Berkefeld filtration
Absent	RIII X	Bearing RIII transplantable breast tumor (M.A. present)		3. Sediment from ultracentrifugation
	C57	Normal Bearing C57 transplantable breast tumor (M.A. present) Bearing C57 transplantable breast tumor (M.A. absent)	C3H	4. 2% saline extract 5. Sediment from ultracentrifugation
			C57 (M.A. absent)	6. 2% saline extract
Not known	Swiss	Normal Immunized with RIII milk agent		

TABLE 16

COMPLEMENT FIXATION TEST WITH RABBIT ANTI-MOUSE MILK AGENT SERUM

<i>General description of antigen</i>	<i>Detailed description of antigen (titre)</i>	
Mouse milk agent	Extract for immunization (32)	
Breast tumors with milk agent	C3H (3, 8), RIII (6, 40), Strong A (28), C57 X (8, 12)	
Breast tumors without milk agent	C57 (2, 8, 16), JK (16), C57 X (14)	
Normal tissues with milk agent	RIII	Lactating breast (32, 16), Spleen (3.5, 10)
	and C3H	Kidney (1.75, 3.5), Lung (1.25, 7), Liver (negative)
Normal tissues without milk agent	RIII X	Lactating breast, (32, 32), Spleen (negative, 1.5)
	and C57	Kidney (1.5, 3), Lung (negative, 1.25), Liver (negative)
Milk	C3H (trace), C57 (negative)	

The behavior of rabbit sera immunized with ultracentrifugal deposits of mouse breast tumor extracts is summarized in TABLE 16, which gives the description of the antigens employed in testing these sera and the various titres obtained in complement fixation tests between these sera and the dif-

ferent antigens. All the antigens used for testing the immune sera, except the material used for immunization, were 2 per cent saline extracts of dried tissues treated with chloroform before extraction in saline. As can be seen, rabbit immune sera gave a strong reaction with the antigen used for immunization. Strong reaction also occurred with extracts of tumors, however, both with and without the agent, as well as with extracts of different normal tissues of agent-carrying and agent-free mice of genetically identical and also of other strains. Lactating mammary tissues of mice with and without the milk agent showed particularly high titre equal to that of the material used for immunization. These results show clearly that the antibody is not specific for the milk agent. The weak titre of the milks used for testing is of interest, which may be explained by the fact that even the high-cancer-strain milk used for testing contained only a small number of the particles and therefore contained insufficient material to give a positive reaction.

TABLE 17
COMPLEMENT FIXATION TEST WITH RABBIT ANTI-HUMAN TUMOR EXTRACT SERUM

<i>Material</i>	<i>Presence of particles</i>	<i>Antigen titre</i>
Breast tumor extract for immunization	+++	10
Human breast tumors	+++ ++ + —	1.75 5 to 6 7 1.75 to 2.5
Human cancer milk	± to +++	Negative
Human normal milk	± to ++	Negative

The results with sera of rabbits immunized with ultracentrifugal deposits of extracts of human breast tumor tissues are shown in TABLE 17. As can be seen, the sera reacted strongly with the material used for immunization. These sera, when tested against 2 per cent saline extracts of different human breast tumors which had been desiccated and treated with chloroform, showed no relation between the number of milk agent-like particles in the extracts and the antigen titre. These sera reacted with all extracts of human breast tumors (even in the case of the extract which showed no particles in the electron microscope). There was no reaction with extracts of human milk, whether of normal women or women with breast cancer. This lack of reaction with extracts of milks of women with breast cancer may be due either to the tumor specificity of the antibodies or to a smaller number of particles actually present in these milks.

The sera of rabbits immunized with ultracentrifugal material from human breast tumor extracts, when tested against mouse mammary tumor extracts, and the rabbit sera against ultracentrifugal deposits from mouse tumor extracts, when tested against human breast tumor extracts, showed strict

species specificity and no evidence of a common antigen in human and mouse breast tumor extracts.

Since the milk agent preparations, largely freed of other material by differential centrifugation, were found to give a stronger reaction with the rabbit immune sera produced by similar material than crude extracts of these tissues with the same sera, the milk agent itself is in part probably responsible for the complement fixation. The agent, however, as already shown, is serologically similar to material present in various tissues free of the agent. This may be due to the structure of particles being composed to some, perhaps not inconsiderable, extent of normal tissue components.

Even apparent physical homogeneity and characteristic particle size are not sufficient for the assumption of chemical and serological differences suggested by the name of virus given to the isolated particles. Cohen (1944) demonstrated that preparations of influenza virus particles contain between 27–57 per cent of normal tissue particles. Knight (1946) estimated from qualitative precipitation data that the most highly purified influenza virus preparations contain at least 20–30 per cent of an antigenic structure characteristic of the sedimentable proteins of normal tissues of the host in which the virus was cultivated. There is no doubt that the purification of animal viruses, in the present state of our knowledge, is a much more difficult, if not impossible task, compared with the purification of plant viruses, if one considers, for example, the purified tobacco mosaic preparations which give no indication by serological or even anaphylactic tests of the presence of normal antigenic structures (Bawden and Pirie, 1937).

In the case of other tumor-inducing agents, the similarity between the Rous agent and normal fowl proteins was shown by complement fixation (Dmochowski, 1948), precipitation tests (Kabat and Furth, 1940), and also by neutralization tests (Gye and Purdy, 1933). In fowl leukosis, preparations of the agent reacted in precipitation tests with antisera to material from normal host tissues, although these sera did not neutralize the agent (Kabat and Furth, 1941). Similarly, the influenza virus was not neutralized by antisera to normal host material, although these sera gave a high titre precipitation test with the virus preparations (Knight, 1946). Similar results have now been recorded with the milk agent preparations. While antisera to normal mouse tissues free of the milk agent induced in one species of animals have shown no neutralizing effect on the agent (Imagawa *et al.*, 1951), antisera to the same material (Imagawa *et al.*, 1951) or similar material (Law and Malmgren, 1951) elicited in another species have neutralized the agent. Although precipitation tests between immune sera against agent-free normal mouse tissues and preparations of the milk agent have shown little antigenic similarity between the agent and normal proteins of mouse tissues (Imagawa *et al.*, 1948), complement fixation tests, described in the present experiments, have demonstrated serological similarity between agent-free tissues and preparations of the agent. Similar results in complement fixation tests have also been recorded with mitochondrial (Malmgren and Bennison, 1950) and with microsome fractions (Malmgren *et al.*, 1951) from normal and tumor tissues of agent-free and agent-carrying mice. Any final conclusions must obviously be based on the results of tests

with sera induced in more than one species of animals by the same preparations of the agent. These sera will have to be examined in all three tests with material from normal tissues of both agent-free and agent-carrying, genetically identical, mice.

The present attempts at isolation of the milk agent, as well as those reported by other workers, show that the agent is particulate in nature, although at the moment agreement about its size is lacking. This can only be achieved by a comparison of the various methods of treatment applied to the same tumor material, which may show the best way towards isolation of products of great homogeneity and greatest biological activity. A further study of agent-carrying and agent-free mammary tumor cells *in vitro*, similar to that of Porter and Thompson (1948), may throw additional light on the aggregation of smaller particles into bigger units and their combination with cell components and it may lead to our understanding of the behavior of the milk agent in tumor cells. It seems that a discussion as to whether the agent is a true virus or an enzyme or a cytoplasmic factor is just an argument in terminology, and any work on the milk agent should proceed without regard to this argument. It appears that this is the only way in which the character of the agent, its relationship to normal tissue constituents, and its mode of action may finally be determined.

Summary

A method of treatment of various tissues of high- and low-cancer-strain mice is described and the results are presented of electron microscope investigations combined with biological tests of tissue extracts obtained by this method.

Several ultracentrifugation procedures with extracts of high- and low-cancer-strain tissues are recorded and the results of investigations of the products obtained by these procedures in the electron microscope and in biological tests are discussed in detail.

An intimate association has been found between the tumor-inducing principle and spherical particles of between 200–300 Å diameter, present in extracts of high-cancer-strain tissues. Preparations containing these particles have shown a comparatively high activity.

These investigations have also shown a serological similarity between the typical particles in tissues of agent-carrying mice and extracts of tissues of agent-free mice.

The same method of treatment applied to human breast tumors and milks of women with breast cancer as well as to milks of apparently healthy women has shown the presence of similar, slightly larger particles, in the majority of the examined tissues with the preponderance in number in tissues and milks from cancer patients.

Immunological investigations have shown no antigenic similarity between particles in human tissues and those in mouse tissues.

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PLANT VIRUS TUMORS*

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Introduction. Do plants have cancer? Is the answer simple?

It is possible that the differences between plant and animal tumors may be related more to differences in the normal histogenesis in the two groups than to differences in their inciting agents or in the basic mechanism or mechanisms of neoplasia.⁵ The identification of plant tumors only with benign teratomas in animals, which they resemble in their possession of many cell types and their lack of malignancy, may be correct or it may be only superficially correct. The various degrees to which the different cells of a tumor are organized with each other and with other cells are probably important in such comparisons. In spite of their abnormalities, the different characteristics of tumor growth in plants and animals retain features of the normal growth characteristics of corresponding healthy cells. The cells that form virus-incited tumors in animals and plants are "sick" cells of types normally existing in the organism. There is no evidence that a virus infection is capable of inducing the formation of a distinctly new cell type foreign to the host.

The multiplication of animal cells induced by viruses is regularly followed by cellular differentiation; such differentiation being the basis for the animal pathologist's descriptive classifications of such tumors. The possibilities of such differentiation are naturally limited to the potentialities of the cells from which the growths start. In animals, the potentialities of the normal generative cells have become limited in various degrees, sometimes to the production of a single cell type. In plants, such limitation of the potentialities of generative or meristematic cells either does not take place or is very limited. The limitation of the potentialities of generative cells in animals is illustrated by the growth of various isolated cell types as such in tissue cultures. The pluripotency of plant generative cells is illustrated by the common, if not complete failure to grow single cell types in tissue culture²⁷ and by regeneration of whole plants from various plant fragments, such as root segments, and pieces of stems or leaves. It is not surprising, therefore, that plant tumors are composed of several cell types, whereas animal tumors may be composed of a single cell type.

Some animal tumor cells penetrate into surrounding tissues and this characteristic makes them more dangerous to the life of the host. In normal growth and development, however, many animal cells penetrate through other tissues and some exhibit amoeboid movements.³² They may assume special relationships with cells from a different germ layer and together with them form organs. Stimuli and responses that control such inter-related growth still find expression in animal tumor development. For example, in the normal development of the breast in the mouse,^{13, 33} the epithelial

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cells grow inward into the mesenchyme and penetrate through it in branching columns, the mesenchyme forming a supporting stroma in the process. The cooperative, almost "symbiotic" development of these two different tissues results in the formation of the breast. When the breast epithelium renews its growth under the influence of the mammary carcinoma virus, any penetration it makes into mesenchyme and any mesenchymal response, forming stroma, would seem to be traceable to normal growth processes of these tissues. Corresponding processes are not characteristic of normal plant growth. The failure to find stroma formation in plant tumor growth should therefore not surprise us.

The most common example of plant cells growing through other tissues of the same plant is that of an embryonic branch root. The mass of cells of the embryonic root grows outward as a unit through the overlying tissues. There is no intimate cooperative growth with overlying tissues, or penetration between these overlying cells. They are crushed, digested or pushed aside as a mass. Invasive or penetrating normal tissue development in plants is extremely rare. Plant organs, generally, develop from masses of embryonic cells which differentiate into the different types in the structure, depending, apparently, on their position within the mass. Plant tumor growth retains, in abnormal fashion, something of this manner of growth. Tumors penetrate other plant tissues but only in mass; there are no finger-like invasive tissue extensions such as characterize certain animal tumors. If haustorial cells of dodder could be rendered tumorous, perhaps a penetrating invasive type of plant tumor would result. There is no basis in normal plant growth, however, for expecting the development of a stroma, even in such an instance.

Some animal tumors metastasize. No plant tumors are known to do so. It is not difficult to understand metastasis in certain types of tumors (lymphomatosis of chickens, for example) where the normal cell prototype meanders through the body. The basis for it is not apparent in the case of tumors, the normal cell prototype of which has a fixed position in some tissue. Why cells of certain tumors should metastasize more frequently to certain locations than to others is also mystifying when the routes of the blood or lymph channels do not afford a ready explanation. The failure, thus far, to find real metastasis in plants may be partially due to the presence of a firm wall around each cell that is cemented to the walls of neighboring cells. The channels of transport in plants, moreover, are inside individual cells, and although sometimes large enough to allow the passage of smaller cells of the same plant, they certainly do not provide the free path that blood and lymph channels furnish for animal cells. The channels for animal cells are much larger and are intercellular in origin. Certain cells not only pass through these channels normally but some even penetrate through their walls normally.³²

In comparing the malignancy of plant tumor diseases with that of animals, it should be pointed out that, in addition to the lack of invasive tumor growth or metastatic tumor growth in plants, plants are, for other reasons, not so vulnerable to tumor growth as animals. An animal has several vital organs, the loss of any one of which will kill it. This is not

true of plants. The loss of a leaf, stem or root usually does not cause death but calls into play regenerative powers of growth which the higher animals lack in any comparable degree.

The writer wishes to suggest for consideration, rather than imply, that differences between plant and animal tumors are related to differences between certain of the life processes of plants and animals, such as their histogenic development, rather than to differences in the basic neoplastic mechanism or mechanisms. Our ignorance in these matters may be comparable to our ignorance of the fundamental sex mechanism before the work of Mendel. Before Mendel's elucidation of the laws of heredity, or before the discovery of the sexual significance of the chromosomes, one may wonder how much resemblance was apparent between the sexual processes in plants and animals.

Virus Tumors in Plants. There are several virus diseases of plants in which leafy outgrowths are produced from the veins. Kroepek disease of tobacco,¹⁶ Smith's rosette of tobacco,³¹ and tobacco mosaic in *Nicotiana paniculata* or *N. tomentosa*¹⁴ may be cited as examples of such diseases. In these instances, the overgrowths are fairly well organized organ-like structures. There are a few plant virus diseases where the overgrowths do not resemble any of the normal plant organs, but consist instead of galls or tumors. Fiji disease of sugar cane,¹⁹ Wallaby-ear disease of corn,³⁰ club-root of tobacco,³⁴ and wound-tumor disease belong in this category. It may be that, in each of these diseases, the overgrowths are capable of indefinite growth as unorganized tissue, but this has been demonstrated to be true only for growths formed in wound-tumor disease. The writer prefers the term "tumors" for the overgrowths of this malady to distinguish their character from that of certain other plant overgrowths, such as many insect galls. The latter, although not constructed like normal plant tissues, are nevertheless highly organized and reach a definite maturity. More is known about wound-tumor than any of the other plant virus tumor diseases and subsequent remarks will be confined to this malady. Crown gall is excluded from the present discussion because a virus etiology for this disease has not been demonstrated. It is also discussed in another paper in this monograph.

Discovery of Wound-Tumor Disease. Wound-tumor virus was discovered in leaf-hoppers of the species *Agalliopsis novella* (Say) collected in the vicinity of Washington, D. C. in 1941. Only 47 individuals of the species were captured. Nevertheless, when the leaf-hoppers were allowed to feed on young crimson clover plants, a few of the plants subsequently developed symptoms of a hitherto-undescribed virus disease. These symptoms consisted of irregularly enlarged leaf veins and the disease was referred to as clover big-vein because of this. The causal virus was named *Aureogenus magnivena* Black since it was thought at that time to be related to the potato yellow-dwarf viruses.¹ At the present time, its relationships are not understood.

Host Range of Wound-Tumor Disease. Insects of the species *Agalliopsis novella* reproduced well on crimson clover plants and, by feeding them on diseased plants, the insects could be used later to determine whether or not other species of plants were susceptible to the disease. A survey of ap-

proximately 100 readily accessible species of cultivated plants and weeds grown from seed revealed that 43 of them in 20 plant families produced symptoms typical of the disease. It is obvious that many more plants must be susceptible.

Morphological Symptoms of Wound-Tumor Disease. The most constant symptoms of wound-tumor were irregular enlargement of the veins and woody tumors on the roots. Other symptoms, observed in one or another of the various hosts, included leaf curling and distortion, leafy outgrowths from the undersides of the veins, vein tumors, distortion of petioles, shortening of internodes, thickening of the stems, suppression of flowers, and dwarfing. All vein overgrowths were on the under sides of the leaves. The



FIGURE 1. Growths incited by wound-tumor virus on a sweet clover stem belonging to clone C10(0.5 \times). Photograph by J. A. Carlile.

FIGURE 2. Tumors induced by the virus on the sweet clover roots of clone C2(0.5 \times). Photograph by J. A. Carlile.

severity of the symptoms ranged from those that were barely detectable to those that were extremely pronounced. In *Portulaca oleraceae* L., the tops of infected plants were indistinguishable from those of healthy plants, although the roots bore numerous small tumors. In certain other cases the symptoms were severe enough to cause the death of the plant. Wound-tumor is one of the minority of plant virus infections that does not cause clearing of the veins as a primary symptom. Of the 43 hosts discovered, the two which produced the most vigorous tumors were cultivated sorrel, *Rumex acetosa* L., and sweet clover, *Melilotus alba* Desr. The latter host, although rarely producing any noticeable vein enlargement in the leaves, developed tumors on the stems as well as on the roots (FIGURES 1 and 2). Tumors were the only conspicuous symptom. These two hosts, *R. acetosa* and *M. alba*, have been most extensively employed in subsequent studies on tumors as such, while crimson clover, *Trifolium incarnatum* L., because

of its suitability as a food plant for the insect vectors and because of its high susceptibility and uniform reaction, has been used as a test plant for the virus.²

Histological Symptoms of Wound-Tumor Virus. The root tumors of sweet clover were discovered to originate in the pericycle opposite the primary phloem and immediately adjacent to the endodermis. In the early stages, the tumor followed a definite and regular pattern of development. Abnormal phloem tissue differentiated first in the basal or innermost portion of the young tumor and this was followed by the differentiation of the apical or outermost cells into abnormal xylem elements. These tracheids were of irregular shapes and sizes and differentiated from the tip towards the base in finger-like processes. Further growth of the tumor from the apex was impossible since the apex was capped by tracheids. The meristematic region between the xylem and phloem and between the xylem extensions continued growing and often pushed up and over the differentiated xylem apex. The subsequent growth pattern was not unraveled. Well developed tumors contained groups of tracheids, some of which were connected to other groups by ramifying tracheids of the same kind. The xylem was surrounded by parenchymatous or meristematic cells through which small branches of abnormal phloem were interspersed. The tumors did not contain the most specialized vascular elements, tracheae and sieve tubes, but, in different species, they did contain relatively the same amounts of vascular and parenchymatous elements as the normal root of the species concerned.

It was determined that some of the stem tumors of sweet clover also originated in the pericycle. The pericycle is one of the fundamental undifferentiated tissues of the stem and root.

Not all of the overgrowths incited by the wound-tumor virus began in the pericycle, however. Small tumors were observed to develop from the cork cambium in roots of *Rumex acetosa*. In certain other plants, there were obvious overgrowths from cambial tissues and, in others, from the phloem of the leaf veins.¹⁵

Cytological Symptoms of Wound-Tumor Disease. The tumor cells in a number of plant species frequently contained many spherical cytoplasmic inclusions which have been named spherules.¹⁷ These have been extensively investigated in sorrel plants. The spherules did not occur in the meristematic cells of healthy plants nor in non-tumorous cells adjacent to tumors in diseased plants. The spherules varied in number in different tumors and in different cells and were found most frequently in cells resembling abnormal protophloem. They ranged in size up to 4μ in diameter. They generally appeared to be solid but sometimes they were vacuolate. The spherules were not dissolved by fat solvents nor stained by Sudan IV but were demonstrably rich in arginine. Safranin, pyronin, and fuchsin stained the spherules as they did the nucleoli, and both were Feulgen negative. The spherules and nucleoli, however, were differentially stained by a nitrous acid-ribonuclease-azure A treatment. In spite of this difference in staining, the spherules may be of nucleolar origin.

Transmission of the Virus. Wound-tumor virus has never been transmitted by any methods involving the transfer of extracted juice from dis-

eased to healthy plants. Practically all of the leafhopper-borne plant viruses behave in this way. In the absence of vectors, plants may be grown in intimate contact without danger of the disease spreading. The virus can be transmitted if a small piece of tumor tissue or an apparently symptomless scion from an infected plant is grafted onto a healthy plant. Three agallian leafhoppers are known to transmit the virus: *Agalliopsis novella* (Say), *Agallia constricta* Van Duzee, and *Agallia quadripunctata* (Provancher).¹ Certain other species in these genera probably would transmit, if tested. However, not all agallian leafhoppers transmit as was shown by numerous failures on the part of *Aceratagallia sanguinolenta* (Provancher).¹

Incubation Period in the Vector. After vector insects fed on diseased plants, a minimum period of 13 to 15 days elapsed before they were able to infect plants, although they were maintained at optimum temperatures between 25 and 32.5°C. Since this period was demonstrated to be the minimum, it is safe to assume that the incubation period is longer in many insects, even at optimum temperatures. At lower temperatures, the incubation period was greatly extended, e.g., 30 days at 20°C. The insects did not transmit the virus at temperatures as high as 37°C. Once insects become infective, they may remain so throughout the rest of their lives. Nymphs and adults of both sexes acted as vectors.²¹

Inoculation of Insects by Injection. Injections of juice from infective insects or infected plants into virus-free vectors rendered them infective.²² This is the only method presently available for studying the virus in cell-free solutions. In this technique, the virus-containing juice, after a preliminary low speed centrifugation, was diluted in a neutral saline solution (0.225 M sodium chloride in 0.01 M neutral potassium phosphate buffer) and minute amounts (0.05 to 0.17 μ l) were injected into the abdomens of the insects. The whole operation was carried out at 0°C. which served to preserve the virus and to immobilize and anaesthetize the insects. Virus in injected insects also underwent an incubation period before the insects were able to infect plants.

Multiplication of the Virus in the Insect Vector. Using this technique, it has been demonstrated that this plant virus multiplies in at least one of its insect vectors. A few μ l. of a dilute solution of juice from infective leafhoppers was injected into a group of virus-free leafhoppers that were maintained on Grimm alfalfa (*Medicago sativa* L.) grown in cages free of weeds at about 25°C. Grimm alfalfa has been thoroughly tested and found immune to the virus. A month later, the injected insects were used as a source of virus for injection of a second lot of virus-free insects. After seven such passages, the quantity of virus with which the experiment had been started had been diluted to 10⁻¹⁸. Nevertheless, titrations showed that there was just as much virus present in the seventh lot of insects as in the original source.⁸

Transmission through the Egg of the Insect Vector. Multiplication of the virus in the insect as well as in the plant helps us to understand the long incubation period of the virus in the insect, its specificity of transmission, and the recently demonstrated fact that about one or two per cent of the progeny of viruliferous females received the virus by way of the egg.⁷

Properties of the Virus. The insect injection technique has also been used to demonstrate the filterability of the causal agent of this disease through filters retaining small bacteria, and to elucidate other properties of the virus. Insect juice proved infectious at dilutions as great as 10^{-5} , plant juice at dilutions as great as 10^{-4} . The virus in neutral saline withstood a temperature of 50°C . for 10 minutes but could not withstand the same interval at 60°C . Virus activity was demonstrable in preparations dessicated and stored at 0°C . for one year. Residual virus activity could also be demonstrated after treatments at H-ion concentrations between pH 4 and pH 9 for one hour.¹⁰

The rate at which the causal virus sediments in capillary tubes and in sugar solutions increasing in density from top to bottom indicates⁹ that it has a sedimentation constant (S_w^{20}) of about 600 s.

Hereditary Differences in Plant Reactions. Individuals of some species, like crimson clover, *Trifolium incarnatum* L., exhibited a very uniform reaction to the wound-tumor virus. Individuals in other species, like sweet clover and sorrel, showed considerable variation in their response to infection. Investigations on sweet clover demonstrated that the heredity of the plant affected the number, size, distribution and morphology of the tumors. Some clones were so susceptible that the large tumors were fused together on the roots under conditions favorable for the disease. Other clones were sufficiently resistant so that though they were infected under the same conditions, the tumors were inconspicuous. The hereditary tumor reaction on the stems did not necessarily correspond to that on the roots.⁶

Virus-free Tumors. One of the most susceptible sweet clover stocks was an inbred line that produced an occasional tumor in the absence of the virus. These "spontaneous" tumors were histologically distinct from the tumors incited by the virus, in that they were less disorganized and could also be readily distinguished from tumors induced by crown gall bacteria, *Agrobacterium tumefaciens* (Smith and Townsend) Conn. The fact that the spontaneous tumors apparently occurred preferentially in this susceptible line suggests an innate hereditary tendency to tumor formation that was exacerbated by the virus.¹⁸

Wounds and the Initiation of Tumors. Wounds played an important part in initiating tumors in infected plants.³ Tumors have been observed to develop from accidental wounds, from points of stress, such as the union of branch and stem, and from the points of emergence of lateral roots. Lateral roots always cause wounds as they begin their growth within the mother root and grow out through overlying tissues. A high proportion of root tumors started at these emergence points. Pin punctures through infected stems also started tumors, although they did not do so in corresponding virus-free stems. The younger the infected tissue wounded, the greater the percentage of wounds that gave rise to tumors. The role of wounds made by lateral roots may partially explain the much greater number of root tumors than stem tumors, since the lateral appendages of the stem arise from superficial meristematic masses of cells without wounds of this sort.

Culture of Tumor Tissue. Tissue from tumors on sorrel roots was isolated, Sept. 22, 1944, and grown aseptically on media. Because the cultured tissue

was the first to be isolated from *Rumex*, it was designated "R 1." It doubled in volume about every three weeks⁴ on the original medium and retained its unorganized growth for years. The tissues were tested for bacteria in a series of experiments after 9-11 months of culture and found to be free of such organisms. The virus was demonstrated to be present in the cultured tissue after 14 months of culture. Portions of it were grafted to sorrel roots. Some of the scion fragments subsequently developed into tumors and the stock plants developed systemic symptoms of the disease. These experiments showed that the tissue was capable of indefinite growth as tumor tissue without the differentiation of normal plant organs. This conclusion was confirmed by experiments in which a small slice from a sweet clover tumor was grafted into the stem of a healthy plant. When this scion tumor tissue had grown to a suitable size in its new site, a small slice was taken from it and the operation repeated. After three such transfers, there was every indication that this process could be continued indefinitely.

On Jan. 28, 1947, 28 months after the isolation of the R 1 tissue, a small root covered with root hairs was observed growing from one of the cultured tissue fragments. Efforts to grow this root in culture failed. It may have arisen because a portion of the tissue grew free of virus. The occasional occurrence of virus-free cuttings from infected sweet clover plants showed that virus-free tissue may arise from previously infected tissue, under conditions as yet undefined.²⁰ The root may have arisen because a portion of tissue had become occupied by a non-tumefacient mutant of the virus, or for some unknown reason.

Physiological studies of the R 1 tumor tissue have resulted in the discovery of an entirely synthetic and much improved medium for its growth¹² and the definition of the optimum temperature and H-ion concentration for its development.²⁸ The R 1 tissue has an unusually high phosphate requirement and is favored by concentrations of hypoxanthine that inhibit the growth of normal sorrel roots in culture. The effects of many chemicals on the growth of the tissue have been tested.^{23-26, 28, 29} It has been demonstrated that the R 1 tissue secretes an α -amylase, a discovery which has furnished the first clear evidence that proteins can be secreted through the plasma membrane and wall of higher plant cells.¹¹

Some Similarities with Mammary Carcinoma of the Mouse. It is interesting to consider some of the similarities that exist between wound-tumor disease, described in detail above, and the mammary carcinoma of the mouse. A virus plays an important role in tumor production in both diseases, and many genetic factors apparently influence the outcome in both. Other factors, such as wounds or hormones, that affect cell multiplication are also involved in the formation of the tumors. Certain tumors occur in the absence of the virus in both. In the case of the plant disease, however, such tumors are histologically different from those incited by virus.

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VIRUS AND TUMORS IN FISHES

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Introduction

Diseases such as fish pox, lymphocystis and certain papillomatous growths have long been considered to be caused by viruses. The evidence that viruses are the etiological agents in these growths is based primarily on the successive development of the lesions in different individuals of the same species (host specificity), the seasonal appearance and disappearance of the growths, the presence of inclusions in the diseased cells, and on other pathological manifestations. In some instances, experimental transmission to healthy fish has been successfully accomplished, but no definite isolation of the agent by filtration, ultracentrifugation or other recognized means has ever been made. The evidence for including these diseases in the virus category is thus purely circumstantial.

1. Hyperplastic Epidermal Disease or Fish Pox

It should be pointed out first that there is no clinical similarity between fish pox and chicken or mammalian pox. This fish disease has been known since the Middle Ages (Hofer, 1904) and is commonly found in several species, especially in European carps and related cyprinids (tench, ide and bream). The lesions occur on the skin, fins, and eyes and are characterized by numerous milky white or gray, flat, raised, or papillary-like growths. Microscopically, there is an exceptional amount of hyperplasia of the epidermis, with the corial elements not being especially overdeveloped and little or no inflammatory reaction. Schlumberger and Lucké (1948) point out that "On histologic grounds no sharp distinction can be drawn between these lesions and true neoplasms but, clinically, the lesions of pox, unlike true tumors, usually regress and disappear entirely."

Loewenthal (1907) described and illustrated intranuclear and cytoplasmic inclusions in certain stages of the disease. Similar inclusions were also seen and reported by Plehn (1910) who questioned their significance, although she recognized carp-pox as infectious and probably due to an ultramicroscopic organism, transmitted possibly by fish lice (Argulids). Lipschütz (1930), commenting on carp-pox, called attention to Loewenthal's (1907) figure 5-i, which showed intranuclear inclusions strikingly similar to those encountered in herpes.

Recently, Roegner-Aust and Schleich (1951) demonstrated inclusions in touch preparations of carp-pox stained with Victoria Blue (Herzberg, 1934). These inclusions or elementary bodies stained dark purple and were believed to be the same as those seen with the electron microscope in palladium-shadowed material. The particles measured 200 to 300 $m\mu$, a size range roughly corresponding to that of pox inclusions in higher vertebrates. Roegner-Aust and Schleich considered these elementary bodies to be the virus of carp-pox, and possibly of other forms of fish pox.

No successful transmission of fish pox has been reported. Epidemiologically, the evidence seems to indicate that an infectious process is involved. The occurrence and regression of the hyperplasia in the same species of fishes, and in the same lake or pond, year after year, points in that direction. Epidemics of carp-pox have been reported throughout the

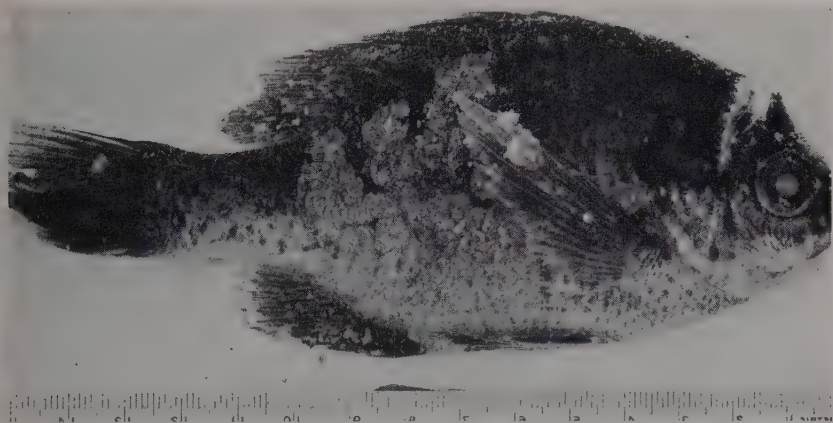


FIGURE 1. Bluegill sunfish (*Lepomis macrochirus*), showing numerous patches of epidermal hyperplasia. Photograph by S. C. Dunton, Photographer of the New York Zoological Society.



FIGURE 2. Enlargement, showing macroscopic features of the epidermal hyperplasia. About 3 X. Photograph by S. C. Dunton.

European continent (Plehn, 1924) but, more often, the disease occurs sporadically, especially in ponds used for carp "farming." The disease is still prevalent in Europe (Roegner-Aust and Schleich, 1951) and has also appeared in several of the newly developed carp "farms" in Israel (Shelubsky, 1950).

An epidermal hyperplasia, pathologically and epidemiologically similar

to carp-pox, was reported by Nigrelli (1948b) in bluegill sunfish (*Lepomis macrochirus*) of Connecticut (FIGURES 1-5). The lesions occurred in the spring and usually had regressed completely by the following winter. In some cases, the growths persisted for more than a year, however, with little or no change in the histological structure (FIGURES 3, 4). Attempts at transmission to healthy fish from other areas were unsuccessful.

Loewenthal (1907) reported that inclusions were not present in the diseased cells of carp, when the epidemics had passed their peak and, at such times, the disease was apparently not infectious. No inclusions were reported in the author's earlier investigations on the bluegill sunfish pox, but a more recent study of early stages of the lesions showed cells with nuclear

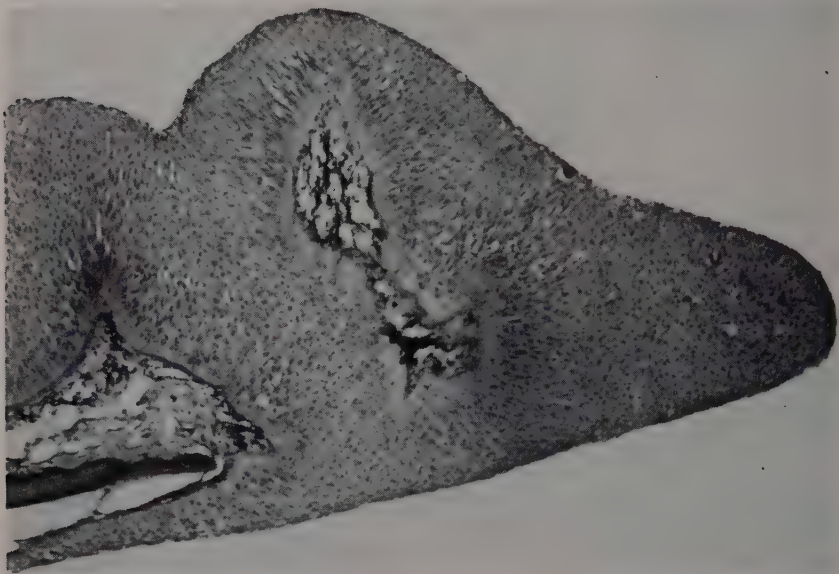


FIGURE 3. Photomicrograph, showing histological picture of the epidermal hyperplasia. Hematoxylin-eosin. 40 X.

aberrations (FIGURE 5) somewhat similar to those reported by Loewenthal (1907).

2. Papillomas

Many of the recorded papillomas of fishes have been described from single cases. Occasionally, however, populations of the same species, living in the same body of water, will develop growths more or less simultaneously or progressively during a particular time or season. The number, size, and gross appearance of the tumors vary considerably but, histologically, they all show the typical papillomatous structure. Inclusions and other pathological manifestations of an infectious process have been found in some instances.

A virus infection was indicated from cytological and epidemiological evi-

dence by Fiebiger (1909) for papillomas in climbing perch (*Anabas scandens*) kept in an aquarium, by Keysselitz (1908) in European barbels (*Barbus fluviatilis*) from the Mosel River, and by Breslauer (1916) in smelts (*Osmerus eperlanus*) from the Baltic Sea. No experimental evidence was obtained which indicated that viruses were involved in the transmission and development of these growths. Fiebiger, however, called attention to the resemblance of the tumors in the climbing perch to infectious warts in mammals.



FIGURE 4. Photomicrograph, showing the relation of the epidermal hyperplasia to the scale and surrounding tissues. Giemsa. 50 X.

Breslauer reported that inclusions were only occasionally found in the tumor cells of smelts.

The tumors in the European barbels were described by Keysselitz (1908) as epitheliomas, but were reclassified as papillomas by Schlumberger and Lucké (1948). Keysselitz traced the development and disappearance of intranuclear (intranucleolar) inclusions and compared the disease to vaccina, epitheliomas of fowls and other virus diseases. He believed that the inclusions represented reaction products of the cell to the invasion of the virus (chlamydozoa). These inclusions were absent in the tumor cells of

fish caught in the summer months, from which Keysselitz inferred that the disease was not infectious at such times.

Recently, three of several climbing perch in the tanks of the New York Aquarium developed papillomas somewhat similar to those described by Fiebiger (1909) for the same species. The tumors were found on the lips, fins and opercular region (FIGURE 6). No inclusion bodies were noted in

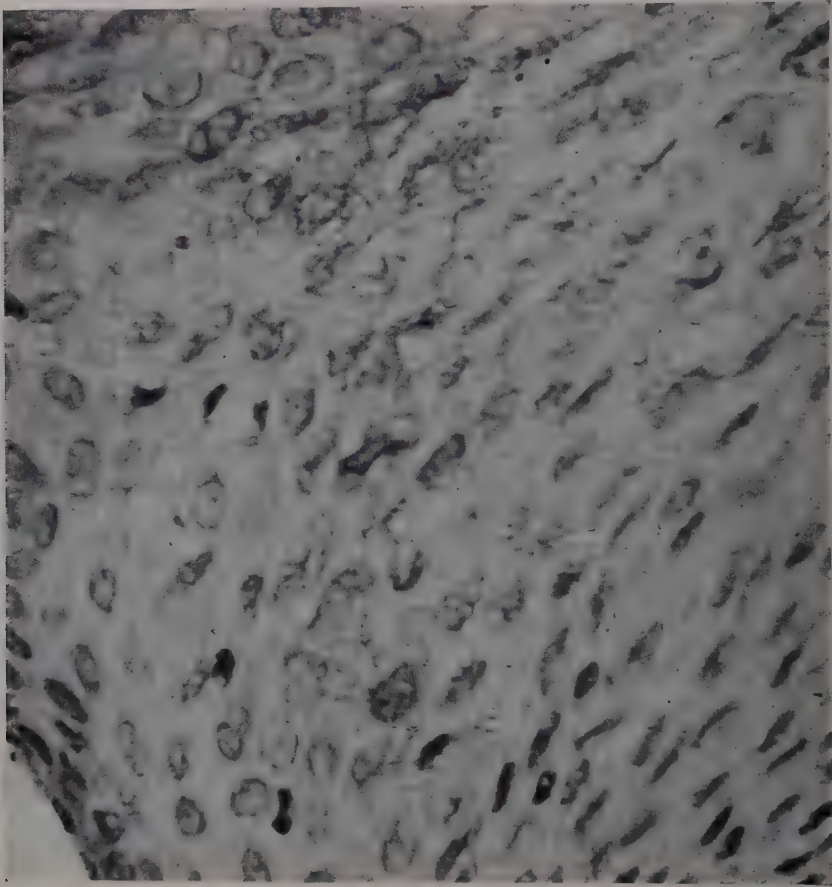


FIGURE 5. Details of the epithelial elements in the epidermal hyperplasia, showing nuclear inclusion similar to those reported by Loewenthal (1907) for carp-pox. Giemsa. 675 X.

the diseased cells and all attempts to transmit the disease by recognized viral methods were without success.

The epidemiology of lip papillomas in the dwarf gourami (*Colisa lalia*) (FIGURE 7) also seems to indicate that an infectious process is present. These fish, raised and bred in outdoor pools in Florida for the tropical fish trade, show a high incidence (0.5–1%) of tumor formation (FIGURE 7). Histologically, the growth shows the typical papillomatous structure, except

that, in many of the cases, there is considerable chondrification and ossification of the supporting tissue. In extreme instances, the growth appears as a malignant papillary epidermoid carcinoma. There is some evidence of inclusion bodies in the epithelial cells but all attempts to transmit the disease under laboratory conditions so far have yielded negative results.

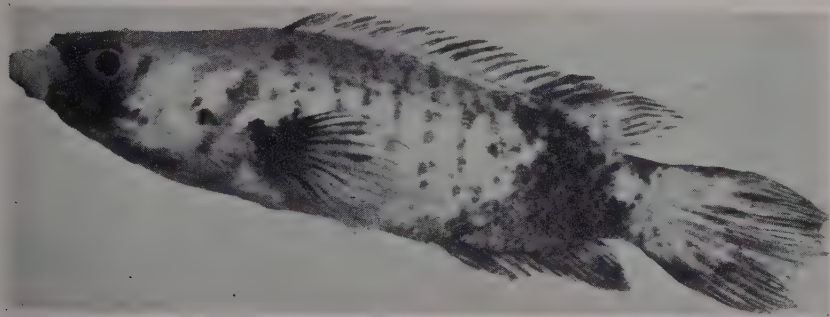


FIGURE 6. Papilloma in the lip of climbing perch (*Anabas scandens*). This is one of three fish which developed such tumors in the New York Aquarium. Fiebiger (1909) compared these growths to infectious warts.

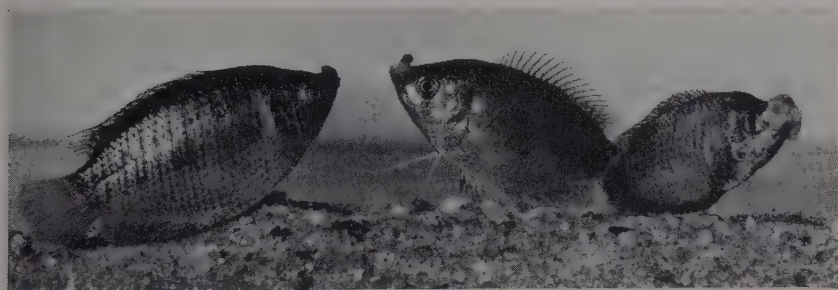


FIGURE 7. Papilloma in the lips of dwarf gourami (*Colisa lalia*). These growths occur frequently in fish bred and raised in outdoor pools in Florida. Photograph by S. C. Dunton.

3. *Lymphosarcoma* in *Esox*

Lymphosarcomas were reported by Nigrelli (1943) in 12 adult pikes (*Esox lucius*) which had succumbed in a tank of the New York Aquarium, at various intervals, during two successive summers. The tumors originated in the kidneys and were characterized by the presence of numerous lymphoblasts supported by a reticular and fibrous stroma (Nigrelli, 1947). Metastases occurred in the liver, spleen and retroperitoneal tissues. Epidemiological studies indicated that an infectious agent was responsible for this disease.

A massive lymphosarcoma was found in the skin around the pelvic region of a muskellunge (*Esox masquinongy*)* (FIGURE 8). Histologically, the growth consisted of lymphoid cells (FIGURES 8-11) supported by connective

* The writer is indebted to Dr. J. F. A. Spret, Department of Parasitology, Ontario Research Foundation, Toronto, for this material.



FIGURE 8. Lymphosarcoma in the skin of muskellunge (*Esox masquinongy*). Photograph by Dr. A. M. Fallis, Director of Parasitology, Ontario Research Foundation, Toronto.

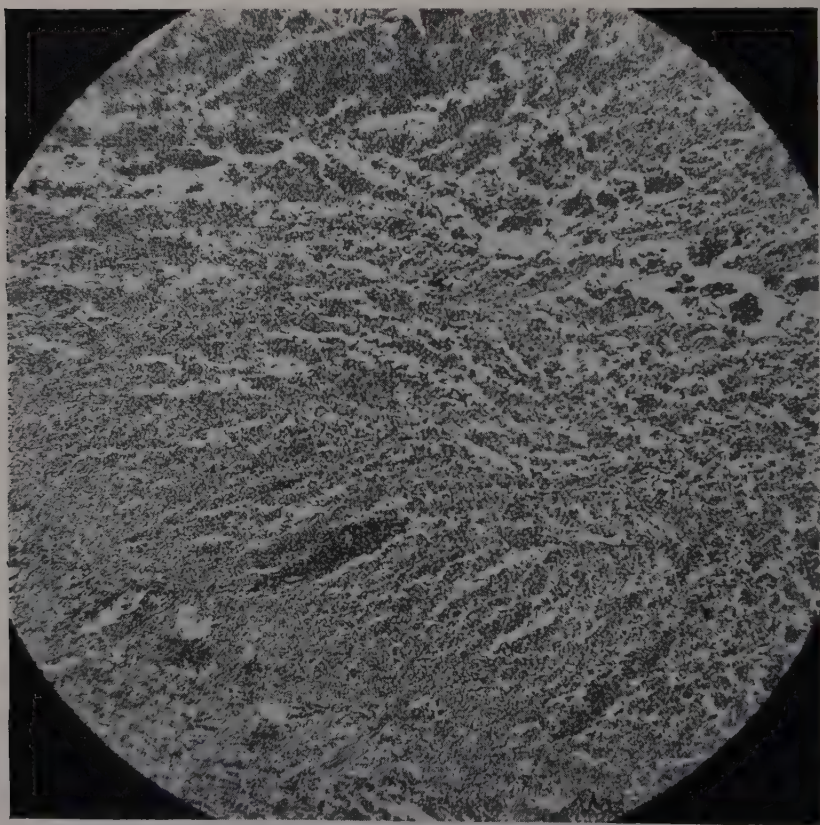


FIGURE 9. Photomicrograph of a section of lymphosarcoma, showing distribution of tumor cells. Hematoxylin-eosin. 75 X.

tissue and a well-developed vascular stroma. There was extensive infiltration into the skin and into the surrounding muscle area. Tumor cells were also found in blood vessels, indicating that metastases had occurred. It is probable that the kidney was the primary site of the tumor. The cells toward the periphery of the tumor were more or less typical lymphoblasts with vesicular nuclei and scanty cytoplasm (FIGURE 10). Those in the

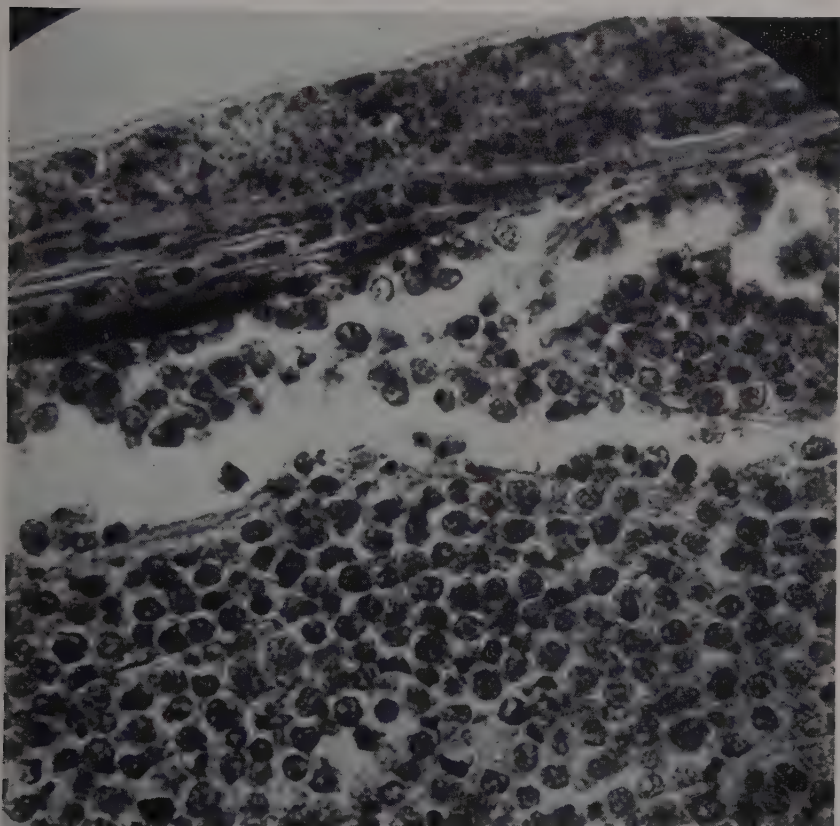


FIGURE 10. Photomicrograph of the lymphosarcoma at the level of the epidermis, showing round lymphoblasts with scanty cytoplasm and vesicular nuclei. Masson's. 675 \times .

deeper parts of the tumor had pycnotic nuclei and the cytoplasm was filled with round and rod-shaped basophilic inclusions (FIGURE 11).

Lymphosarcomas apparently occur frequently in this group of fishes (Esocidae) and the evidence indicates that viruses may be the causative agents.

4. *Lymphocystis Disease or Cell Hypertrophy*

Lymphocystis, a disease of some economic importance in marine and freshwater fishes, has been considered viral in origin by Weissenberg (1914, 1921, 1939, 1951a), Rasin (1927, 1928), Hyde (1937), Smith (1940) and

others. In temperate freshwater fishes, the disease usually appears in the spring and gradually disappears during the summer, although in some cases it may persist for one or two years.

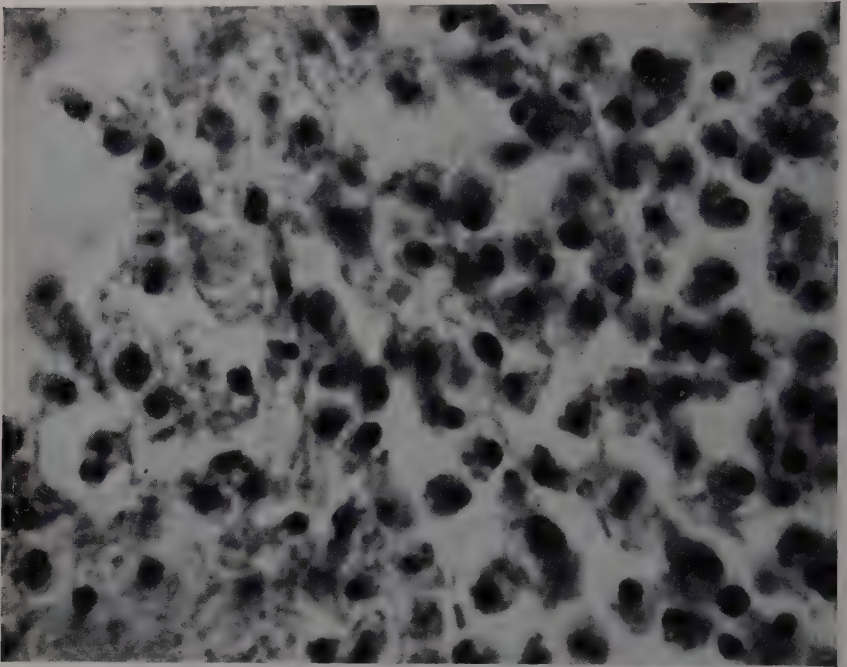


FIGURE 11. Photomicrograph of lymphosarcoma cells with pycnotic nuclei and abundant cytoplasm filled with inclusions. Masson's. 1000 X.

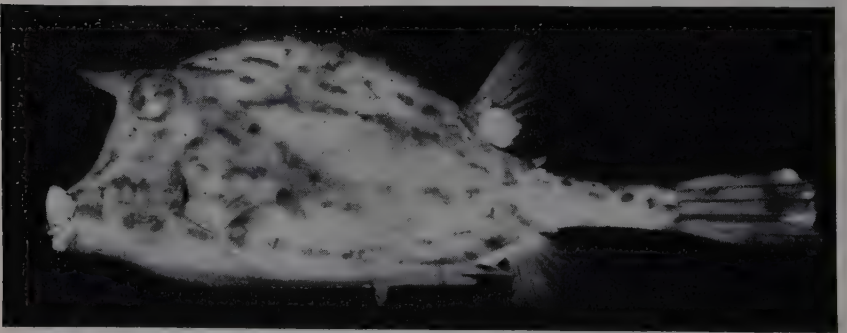


FIGURE 12. West Indian cowfish (*Lactophrys tricornis*) from west coast of Florida, showing lymphocystis tumors on the lip, base of the dorsal fin and tail fin. Note the granular nature of the tumor, each "granule" represents a lymphocystis cell. Photograph by S. C. Dunton.

The lesions (FIGURES 12, 15, 16) are usually grayish nodular or flat growths, shown microscopically to contain ovoid or spherical bodies measuring up to 5000 microns or more, depending on the host species. These

bodies (FIGURES 13, 14), referred to as lymphocystis cells and, showing a striking resemblance to invertebrate eggs, are regarded by Weissenberg as enlarged fibroblastic, osteoblastic or phagocytic cells. They are usually found clustered in lymph spaces of the skin and are sometimes surrounded by lymphocytes and melanophores. In some cases, there is a considerable

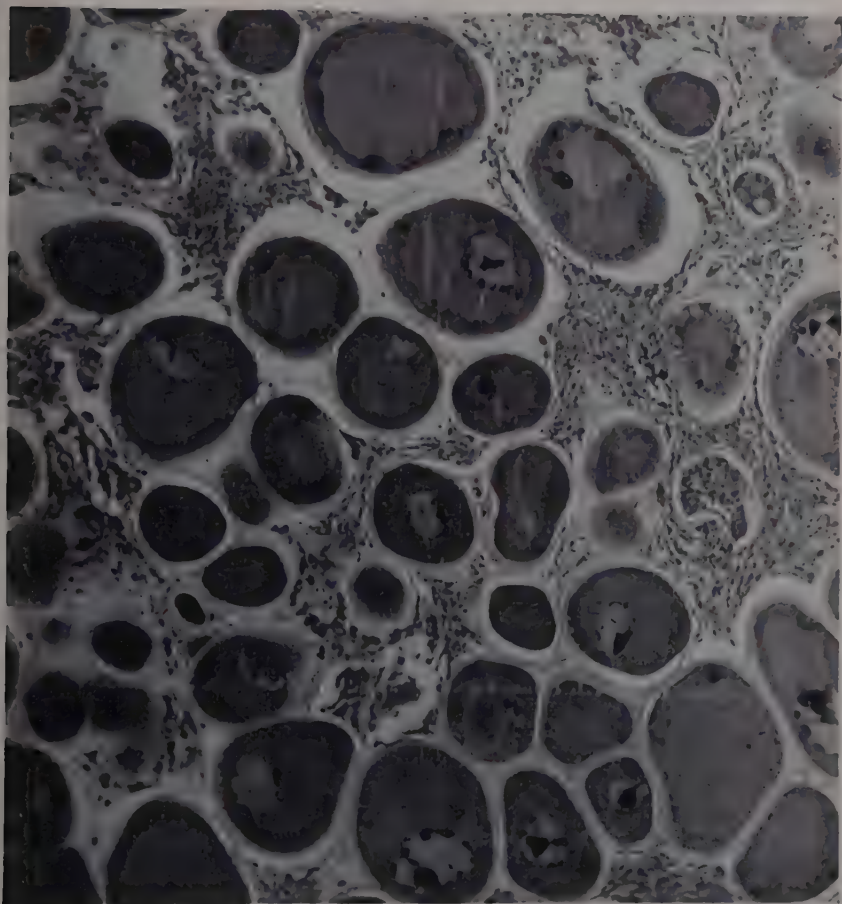


FIGURE 13. Section through the tumor mass of the dorsal fin, showing lymphocystis bodies of various size in lymph spaces. Note so-called cytoplasmic inclusions. Masson's. 50 X.

development of connective tissue, bordering on neoplasia (Walker, 1947). Lymphocystis cells may also be found in the stomach wall, spleen or ovaries (Nigrelli and Smith, 1939), free in the branchial cavity, adhering to the gill epithelium and within the gill filaments (FIGURE 15) (Nigrelli, 1950).

Each lymphocystis element is surrounded by a thick hyalin capsule. The nucleus, with one or more nucleoli, is enlarged and the cytoplasm may contain basophilic, Feulgen-positive granules or a network. Weissenberg (1939), and more recently Alexandrowicz (1951), traced the development of

the inclusions from a single Guarneri-like body in the young lymphocystis cell. The inclusions were described as growing in size with a concomitant increase in volume of the diseased cell. Eventually, the basophilic material develops into a complex network. This structure contains osmiophilic granules, which are considered by Weissenberg (1951a) to be the true virus particles or elementary bodies of lymphocystis.



FIGURE 14. Section of a gill of cowfish with a single lymphocystis body. Note thick capsule around the cell. Masson's, 75 X.

Experimental transmission of the disease to healthy fish has been reported by Weissenberg (1941, 1939, 1945, 1951b, c) and by Rasin (1927, 1928). The latter investigator showed that the disease in paradise fish (*Macropodus* sp.) could be transmitted to healthy fish only if they were bruised before being introduced into a tank of water containing an emulsion of lymphocystis tissue. Rasin further reported that one part in a million of the emulsion was still virulent and that the infective agent was highly resistant to drying. He also succeeded in transmitting the infection to a gourami (*Trichogaster fasciatus*), belonging to the same family. He was unable, however, to demonstrate a filterable agent.

Weissenberg believes that lymphocystis disease is transmitted through the gills and recently (1951b, c) reported a positive inter-ordinal trans-

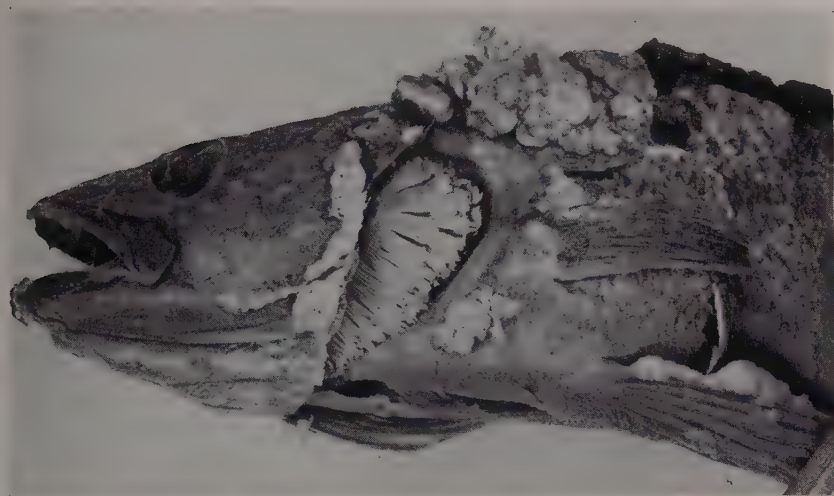


FIGURE 15. Pike-perch (*Stizostedion vitreum*) from Lake Erie with several lymphocystis tumors. Exposed gills show numerous copepod parasites. Photograph by S. C. Dunton.

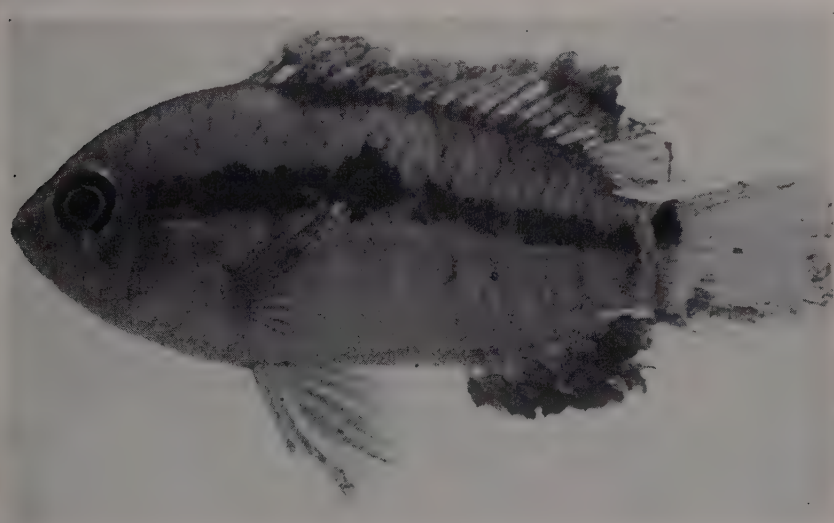


FIGURE 16. Lymphocystis on the fins of the acara (*Aequidens portalegrensis?*). Photograph by S. C. Dunton.

mission of the disease from the pike-perch (*Stizostedion vitreum*) to the killifish (*Fundulus heteroclitus*) with material filtered through a Chamberland-Pasteur filter L-5. This is the first time in the long history of the disease that the filterability of lymphocystis virus has been demonstrated.

The writer, together with the late Dr. George M. Smith of Yale Medical School, has studied lymphocystis disease since 1937 in a number of temperate and tropical freshwater and marine fishes.*

All attempts to transmit the disease under laboratory conditions have heretofore been without success. Lymphocystis disease has recently been discovered in a tropical aquarium fish, provisionally identified as the acara [(*Aequidens portalegrensis* (FIGURE 16.))† The diseased fish were part of a shipment which included a number of other cichlids. Lymphocystis was found on the fins in 30 of about 100 acara and several individuals of another species of *Aequidens*. An analysis of all the species involved in the shipment showed that this infectious disease was limited to the original group, even after two months of observations. The disease did not reappear in those fish in which the lesions were removed by cauterization, a procedure used by Mr. Henry Hessel, when the lymphocystis tumors were small and limited to the fins.

Attempts to transmit the infection to healthy feral or aquarium-bred acara by cohabitation with diseased fish were unsuccessful. Negative results were also obtained when some of the healthy fish were fed freshly teased and emulsified lymphocystis material, part of which was incubated for 4 hrs. at 37°C. In other healthy fish, bits of lymphocystis tissue and cells were implanted under the skin, at the base of the dorsal fin. After several days, there was evidence of infection characterized by massed macrophages which reached a size up to 30 microns, containing melanin and refractile granules. Within 10 days a large mass had developed showing typical lymphocystis cells. Further studies, using the transplantation technique, are now in progress.

All investigators studying lymphocystis disease for the first time are impressed with the striking similarity of the lymphocystis cell to an invertebrate egg cell. Nevertheless, they have concluded that the lymphocystis cell is a true connective tissue element which has undergone the peculiar enlargement and transformation characteristic of the disease.

One interesting feature associated with the disease is the presence of ergasilid (copepod) parasites on the gills, first reported by Nigrelli (1950). There is a striking resemblance between the lymphocystis body (FIGURE 14) and the eggs of these copepods. The degree of parasitic infestation also appears to be correlated with the extent of the lymphocystis disease. Practically all fishes in which lymphocystis has been reported are known to be hosts for these ergasilids or related copepods and the known reproductive cycles of these crustaceans coincide with the appearance of the disease.

When the diseased acara used as a donor for the previously mentioned experiments was sacrificed, its gills were found to be infested with an aquatic mite. Some species of such mites are known to be used as food by fishes, and others habitually lay their eggs in the tissues of mollusks and of

* Blue angelfish, common hogfish, orange filefish, black angelfish, banded butterfly fish, East Indian and West Indian cowfish, clownfish, striped sleeper, orange spotted sunfish, common sunfish, and pike-perch. At present, epidemiological studies on lymphocystis in the striped bass (*Morone saxatilis*) from New York and Connecticut coastal waters are in progress in collaboration with conservation authorities of these states.

† The writer wishes to thank Mr. Edward Weiss (Buccaneer Aquarium, Brooklyn, N. Y.) for these specimens and Mr. Henry Hessel (Roosevelt Aquarium, Brooklyn, N. Y.) for information included in this discussion.

other aquatic animals. Aquatic mites are inhabitants of both fresh (Hydrachnidae) and marine (Halacaridae) waters.

There are still many histological and cytological features of lymphocystis disease which remain to be explained. Some of these are as follows: (1) the exact origin of the lymphocystis cell; (2) the lack of continuity of the diseased cells with the surrounding tissue; (3) the presence of the cells in lymph spaces of the skin and free in the branchial cavity; (4) the presence of a thick (chitinous?) capsule around a supposedly vertebrate fibroblast, osteoblast or phagocytic cell; (5) the enlargement of a vertebrate cell from the usual 10–15 microns up to 5000 microns or more; and (6) the relationship of ectoparasites (argulids, copepods, and water mites) to the disease.

Discussion

The etiology of some of the tumors and hyperplastic growths in fishes is well known. For example, Nigrelli and Smith (1938, 1940) and Nigrelli (1940, 1943, 1948a) reported myxosporidian and microsporidian parasites (Sporozoa) in certain fish tumors. Gordon (1948, 1950) has established the genetic basis of melanomas and other atypical pigment cell growths in live-bearing top-minnows. The possibility that a virus may be involved in these malignant, genetically controlled neoplasias has been suggested.

There are some known virus diseases in fish which do not cause tumors. Pacheco (1935) experimentally showed that epidemics of infectious stomatitis in fishes in some of the river systems of South America were caused by a filterable organism which retained its virulency at 0°C. Torres and Pacheco (1934) demonstrated eosinophilic inclusions in the diseased cells.

Certain inbred races of European carp are susceptible to ascites, an infectious disease occurring during certain seasons. It was formerly believed that this condition was caused by *Pseudomonas punctata* var. *ascitae* (Schäperclaus, 1930, 1939). Recently, however, Roegner-Aust and Schleich (1951) reported that the etiological agent was a virus which could be demonstrated with the electron microscope in palladium-shadowed material prepared from the body cavity exudate, bacteria-free filtrate and ultracentrifuged fractions of the filtrates of the exudate and organ fluids of diseased fish. These elementary bodies, measuring 100 m μ , were considered to be the virus responsible for the disease, and the bacteria were believed to be secondary invaders. When fish were kept at 20°C. and were injected intraperitoneally with the filtrate, the typical Western Europe type (non-ulcerating) of carp ascites was produced and the fish invariably died within a few days.

There is some evidence that the "kidney disease" found in hatchery reared trout and salmon may be due to viruses. Belding and Merrill (1935) reported a chronic infectious disease, causing the deaths of large numbers of hatchery-bred brook trout. The disease, which appeared during the rise in water temperature, was characterized by multiple kidney abscesses and metabolic disturbances of the body fluids. Although a gram-positive, non-capsulated, motile bacillus was found associated with the lesions, inoculation experiments with cultures of this organism were negative. A some-

what similar disease in the blueback salmon was thought to be due to *Pseudomonas*. Rucker (1951), in collaboration with Dr. C. A. Evans of the University of Washington, has indicated, however, that the disease could be produced experimentally by intraperitoneal injections of bacteria-free filtrates of tissue and fluids from diseased fish.

Schäperclaus (1941) reported a highly infectious kidney disease (Nierenschwellung) among rainbow trout in European hatcheries. At one station, 80,000 fingerlings were lost in 14 days. Microscopically, the lesions showed a considerable hyperplasia of the connective tissue of the kidney and necrosis of adjacent structures. Bacteria-free filtrates of emulsified diseased kidney and liver produced typical lesions in almost 100 per cent of the experimental trials. On the basis of the above experiment, Schäperclaus (1941) concluded that the infectious kidney disease in European rainbow trout was the result of a virus.

The above discussion indicates that viruses are the cause of some diseases in fishes. The epidemiological and pathological evidence strongly suggests that similar agents may be involved in certain hyperplastic growths (fish pox) and neoplasia (lymphosarcoma and some papillomas) reported in this paper.

The spontaneous development of tumors, simultaneously, progressively and repeatedly, in large numbers of individuals of the same species of a single population, living under the same conditions, would lead one to believe that an infectious agent was responsible. In the absence of any visible organism, a virus should be suspected and investigated. Some of the piscine neoplastic growths which should be re-investigated along these lines are: (1) thyroid carcinomas and goiters, especially those occurring in hatchery-reared salmonoid fishes, since an infectious agent was suggested by Gaylord and Marsh (1914) and since the exact etiology of these growths in fishes and in humans is still questionable (Greenwald, 1950); (2) lip epithelioma in catfish (*Ameiurus nebulosus*) reported by Lucké and Schlumberger (1941), since the frequency of this tumor is high and since it is one of the few transplantable growths reported in the literature; and (3) mesenchymal tumors of the corium of goldfish described by Lucké, Schlumberger and Breedis (1948), since such growths occur frequently and show a tendency to regress and since the authors suggest that the tumor-inducing factors were either transmissible or environmental. The latter species, together with the dwarf gourami (*Colisa lalia*) reported in the present paper, should make excellent experimental animals, as they are regularly bred and raised in large numbers in pools and aquaria.

Summary

Virus diseases in fishes do occur, but the evidence that they are the cause of hyperplastic and neoplastic growths is purely circumstantial, being based chiefly on epidemiological and pathological studies. The following diseases which have been considered viral in origin are discussed: fish-pox, fish papillomas, certain lymphosarcomas, and lymphocystis (cell hypertrophy). A successful transplantation of lymphocystis tissue in acara (cichlid) is reported.

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KIDNEY CARCINOMA IN THE LEOPARD FROG: A VIRUS TUMOR*

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In the leopard frog (*Rana pipiens*), the kidney is frequently the site of a malignant tumor, an adenocarcinoma.¹ In a survey of over 10,000 frogs made ten years ago, the incidence of this tumor was 2.7 per cent.² While some variation in frequency occurred in different lots, the incidence remained about the same. In more recent surveys, based upon frogs from the northern New England States and the adjacent parts of Canada, a similar high incidence has been found. No accurate figures as to incidence in frogs from other regions are available, but tumor-bearing frogs have been obtained in considerable numbers from the Mississippi Valley, North Dakota and Indiana. To date, 1429 frogs with naturally-occurring tumors have been studied in this laboratory. The tumors were more prevalent in males than in females; 1012 (70.8 per cent) of the tumor-bearing frogs were males, 417 (29.2 per cent) were females. An analysis of the series with respect to location and number of tumors is given in TABLE 1, and shows that both kidneys were more frequently involved than either kidney alone. Bilateral tumors were commonly multiple, but solitary tumors predominated when only one kidney was involved.

Evidence that the Frog Kidney Tumor is a True Neoplasm

In a symposium dealing with viruses as causative agents in cancer, it seems of primary importance to demonstrate that any tumor under discussion does, in fact, belong in the category of cancer and that it is a true malignant neoplasm, not a tumor-like lesion such as an infectious or regenerative hyperplasia. The frog tumor fulfills all the criteria by which one recognizes a tumor as a malignant neoplasm, whether in man or in other vertebrates; for it invades and destroys the tissue of its origin; it metastasizes; it can be serially transplanted; and the activities of certain of its enzymes are altered in a manner similar to that of mammalian cancers. These several characteristics of the frog carcinoma will now be reviewed briefly.

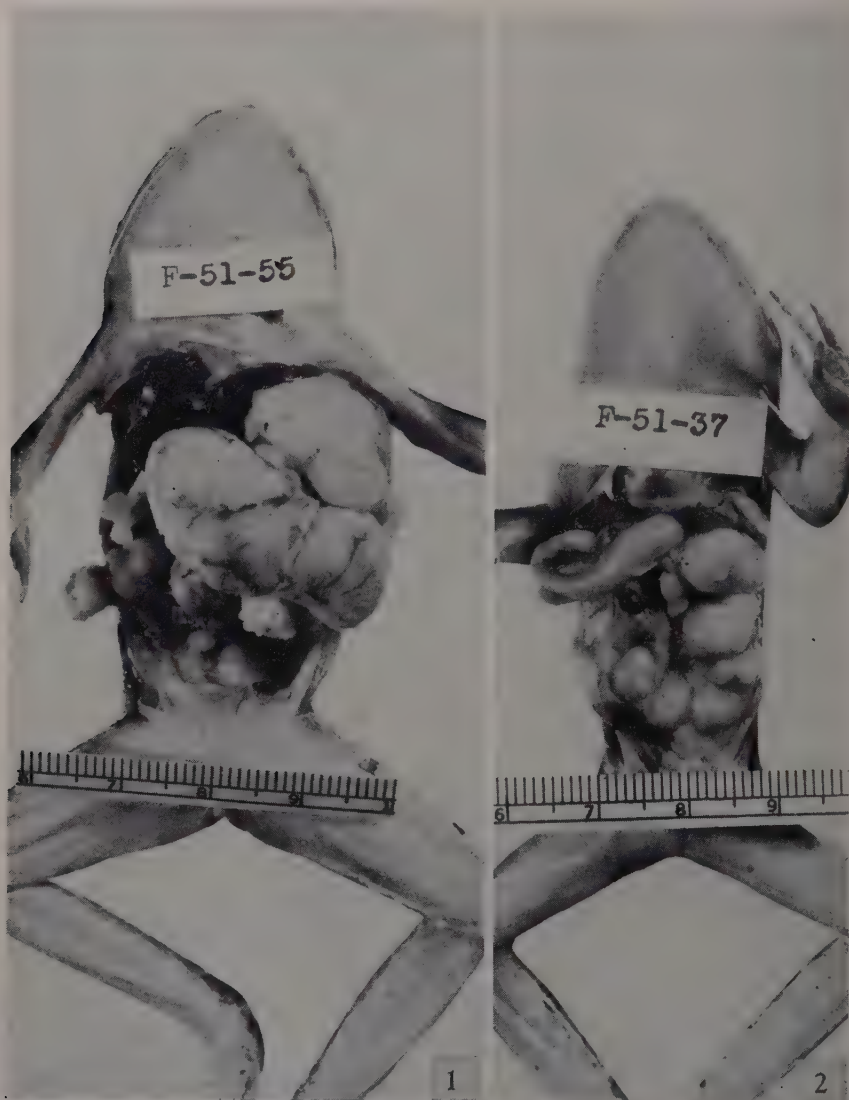
Gross and Microscopic Appearances. The frog tumors are ivory-white in color, contrasting well with the brownish renal tissue. Most of them are somewhat firmer than the surrounding normal kidney. They range in size from tiny, early tumors to large masses that have destroyed all but small portions of the kidney, displacing the neighboring organs and nearly filling the coelomic cavity (FIGURES 1 and 2). Such large tumors are common, but the mean size, in unselected series of naturally-occurring tumors, is 4 to 6 mm., that is, approximately one-third to one-half the size of the normal kidney.

As shown in TABLE 1, about one-half of the tumors are solitary; the other half are multiple. The different modes of origin are exemplified in FIGURES

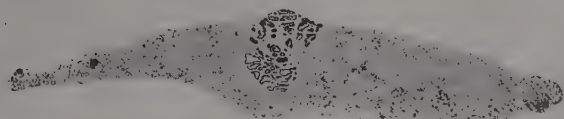
* This investigation has been aided in part by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

TABLE 1
NUMBER OF NATURALLY-OCCURRING RENAL CARCINOMAS IN LEOPARD FROGS

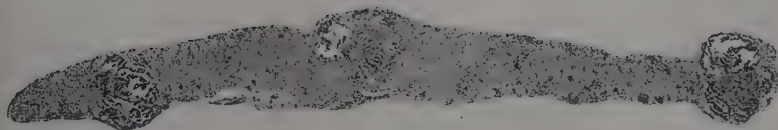
	<i>Number of frogs with solitary tumor</i>	<i>Number of frogs with multiple tumors</i>	<i>Total number of frogs with tumors</i>	<i>Per cent of grand total of 1429 frogs</i>
Both kidneys involved.....	345	568	913	63.9
Right kidney alone.....	189	70	259	18.1
Left kidney alone.....	179	78	257	18.0
Total.....	713	716	1429	100.0



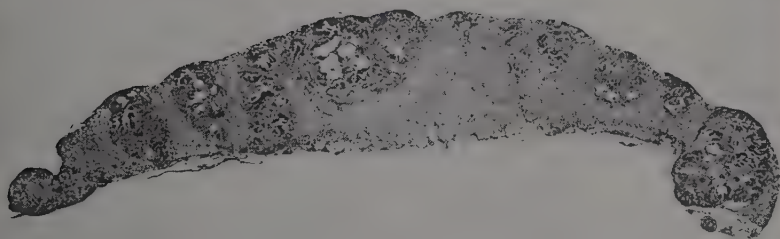
FIGURES 1, 2. Large bilateral renal tumors that have destroyed all but small remnants of the kidneys. The tumors fill the greater portion of the coelomic cavity, displacing the organs.



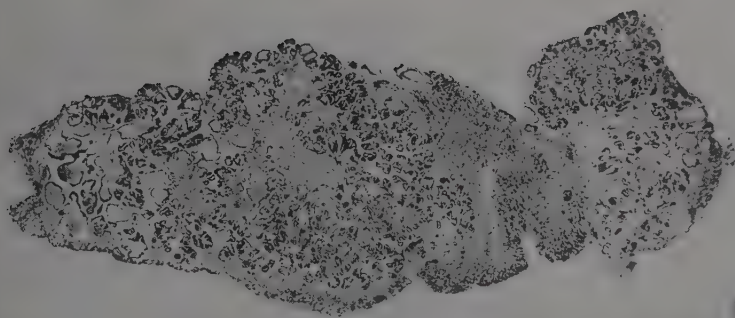
3



4



5



6

FIGURES 3-6. Longitudinal sections through four entire kidneys to show appearance of relatively small tumors at low magnification. The tumors may be solitary (FIGURE 3) or multiple (FIGURES 4-6). The tumor in FIGURE 6 has destroyed the major part of the kidney. Magnification: 7X.

3 to 6, which are longitudinal sections through entire tumor-bearing kidneys, shown here at a low magnification. FIGURE 3 illustrates a solitary tumor. FIGURE 4, by contrast, shows three tumors in different parts of the kidney.

The nearly equal size of these tumors suggests simultaneous development from several foci. Serial sections of many such kidneys have failed to bring out any communications between the individual tumors, indicating they have arisen multicentrically. More extensive multicentric involvement of kidneys will be noted in FIGURES 5 and 6.

Microscopically, the majority of tumors have the appearance of typical adenocarcinomas, in this way resembling the most common variety of cancer in man. They are composed of epithelial cells that are much larger and more basophilic than normal kidney cells. Usually these cells are arranged in several layers around irregularly shaped lumina, into many of which papillary projections extend (FIGURE 7). Mitotic figures are numerous, as a rule, and frequently several may be seen in a single neoplastic "tubule." The stroma is scant and poorly vascular. A capsule is lacking, and extensions infiltrate and destroy the adjacent kidney at the margin of the tumor (FIGURE 8). In a relatively small group of the tumors, the component cells are less atypical and arranged in a more orderly fashion. These growths resemble adenomas rather than adenocarcinomas. Commonly, such growths are cystic in parts, with pronounced papillary ingrowths. All gradations are found between the frankly malignant, invasive and destructive adenocarcinoma and the structurally benign adenoma, cystadenoma and papillary cystadenoma. In general, the larger tumors have a malignant appearance, although many of the minute nodules are also obviously carcinomatous. Once established, the neoplastic disease appears to be progressive, for tumors with evidence of regression, such as atrophy of cells, extensive necrosis and conspicuous overgrowth of stroma, are uncommon. It is probable that all these tumors are at least potentially cancerous and that they go through evolutionary phases in their progress toward ultimate malignancy. The general structure and behavior of the frog kidney tumor have their counterparts in the renal adenocarcinoma of man.

The outstanding characteristic of this frog tumor is the frequent presence of acidophilic intranuclear inclusions which, in general appearance, are like those found in herpes and certain other diseases known to be caused by viruses (FIGURE 9). In their typical, fully developed form, the inclusions are readily recognizable and they were observed in such form in over one-half of the present series. It is obvious that there must be developmental stages of the inclusions, but the appearance of the early stages is still a matter of uncertainty. Moreover, there appear to be seasonal variations; at least, fully developed inclusions in the tumor are more frequently encountered in winter and spring than in summer and autumn. The inclusions are invariably confined to the neoplastic cells. They have never been observed in normal renal epithelium of tumor-bearing kidneys, or in the normal cells of other organs. The number of inclusions in the neoplastic tissue varies greatly; sometimes, relatively few are present; sometimes, nearly every cell in some portion of the tumor is affected.

Metastasis. The cardinal sign of malignancy is metastasis. The fact that the frog tumor possesses the potentiality to metastasize is clear proof

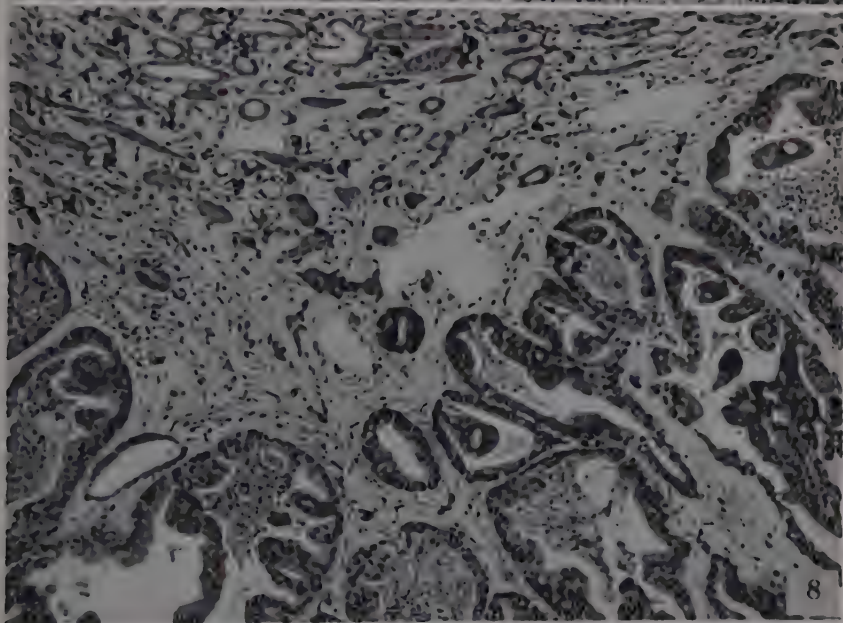
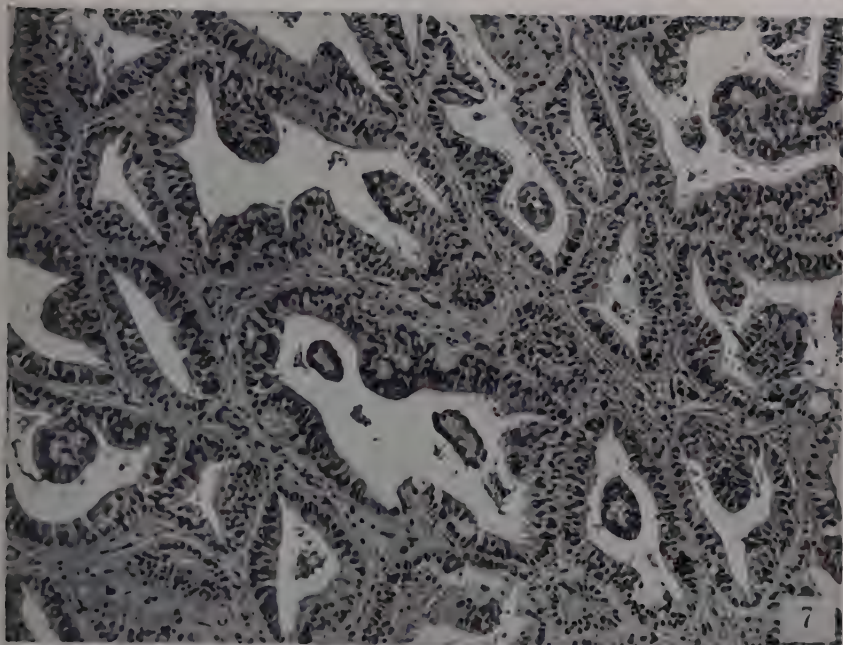


FIGURE 7. Microscopic appearance representative of the majority of tumors. The tumor is composed of large gland-like structures that are lined by one layer and have several of irregular epithelial cells. In many areas papillary projections extend into the lumina. The stroma is delicate. Magnification: 180X.

FIGURE 8. Margin of a tumor showing absence of encapsulation and invasion of the renal tissue. Magnification: 100X.

of its being a cancer. Like many kinds of human cancer, including "hypernephroid" carcinoma, the frog tumor usually becomes disseminated

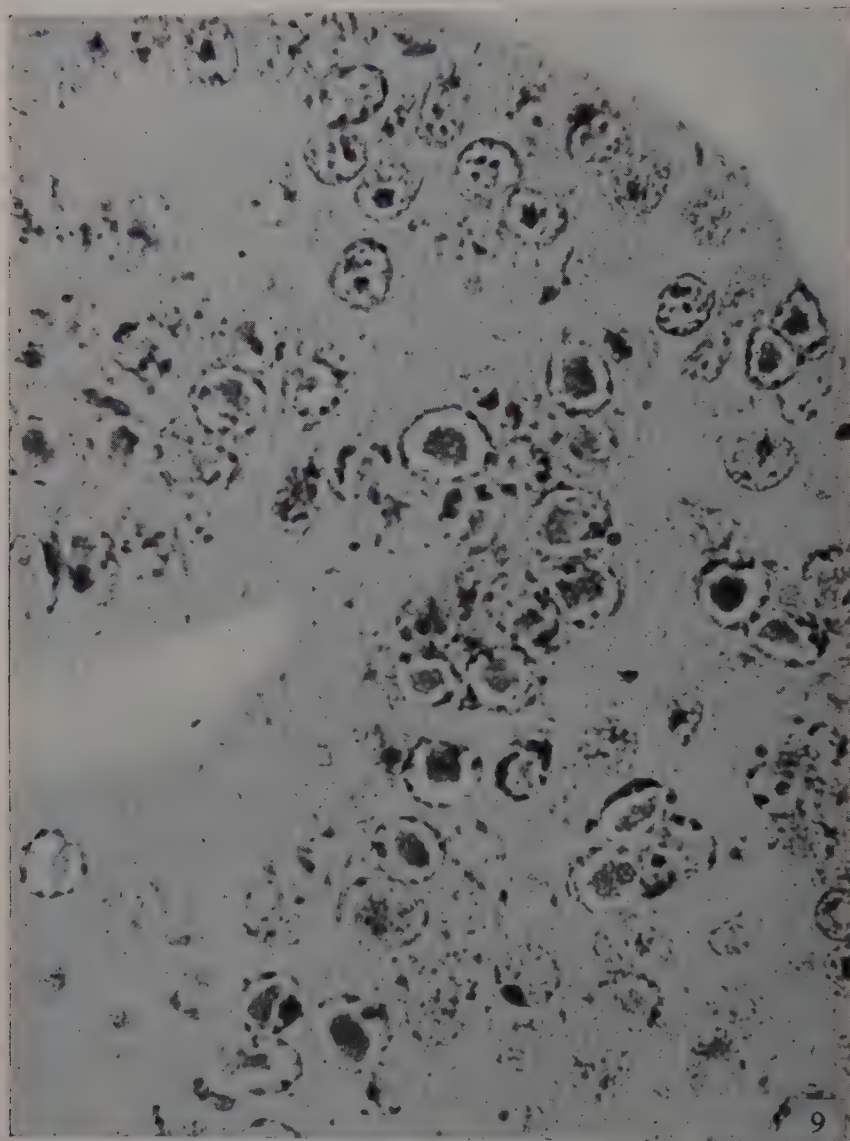


FIGURE 9. Intranuclear inclusions in neoplastic cells. In this field most of the nuclei are swollen and contain prominent acidophilic inclusions. There is usually a clear space between the inclusion and the margined chromatin. Magnification: 1200X.

only after it has attained considerable size.³ Definite figures as to the incidence of metastasis would have little meaning, since the great majority

of the tumor-bearing frogs in this series were examined when the tumors were still small. Suffice it to state that more than 100 instances of metastasis have been encountered. Approximately three-fourths of these were in frogs that had either been living under outdoor, natural conditions or kept under as natural conditions as possible in a cool amphibian vivarium. When, for purposes of experiment, tumor-bearing frogs were exposed for approximately 50 days to a constant high temperature (28°C.), over one-half of the animals developed secondary and usually exceptionally numerous⁴ growths (FIGURE 10).

Dissemination of the tumor takes place most commonly through the vascular channels and, upon microscopic examination, tumor-cell emboli are often found even in cases in which metastatic growths have not as yet become established (FIGURES 11–13). The liver and lungs are the most frequent sites of metastatic growths, but various other organs may also be involved. Stages in the development of metastases through growth of tumor emboli are shown in FIGURES 14 to 17. The great extent of involvement that is observed at times is illustrated in FIGURES 18 and 19.

Serial Transplantation. It is well known that malignant neoplasms can be transplanted far more readily than can their non-malignant counterparts. In many experiments, because of interest in factors that affect the manner of growth of cancer, the anterior chamber of the eye was chosen as the site for transplantation. Most frog tumors (about three out of four) readily become established here and about one-half of the transplants grow until they completely fill the anterior chamber.⁵ More than 60 frog tumors in all have been successfully transplanted to this site. Once established, the tumors can be serially transplanted. Thus, in one experiment, a frog tumor was propagated for two and one-quarter years through fourteen generations.⁶

Biochemical Differences that Distinguish the Frog Carcinoma from Normal Renal Tissue. Lately, there has been a clarification of the differences in enzyme activities of cancers as contrasted with those of the tissues of their origin. For certain enzyme systems, such differences appear so distinctive that they can be included among the characteristics of cancer.⁷ Our studies have been largely confined to catalase and the phosphatases. These enzymes were chosen because their activities are high in the normal kidney and because they are known to be greatly reduced in the mammalian cancers hitherto investigated. Similar changes are found in the frog carcinoma. Catalase activity in these tumors is reduced, on the average, to about one-tenth of that of the normal kidney.⁸ Moreover, as in mammalian cancers, catalase is also considerably reduced in the non-involved liver and, when minute amounts of frog tumor homogenates are injected into the coelomic cavity of normal frogs, prompt reduction of liver catalase results.⁸

The phosphatases were studied over a pH range of from 4 to 11. Over the entire range, the phosphatase activity of all frog carcinomas was profoundly diminished.⁹ It may be concluded that the cells of the frog tumor undergo changes in catalase and phosphatase analogous to those of mammalian cancers.

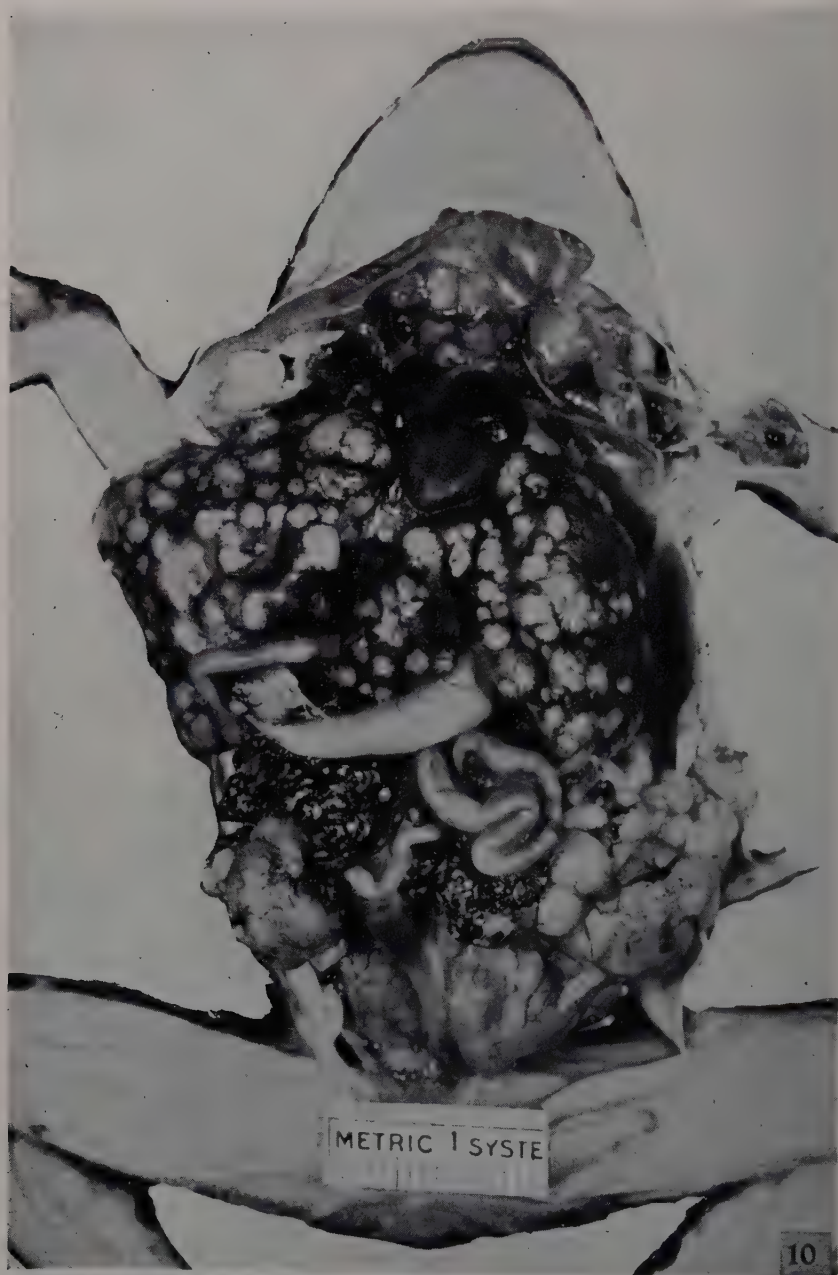
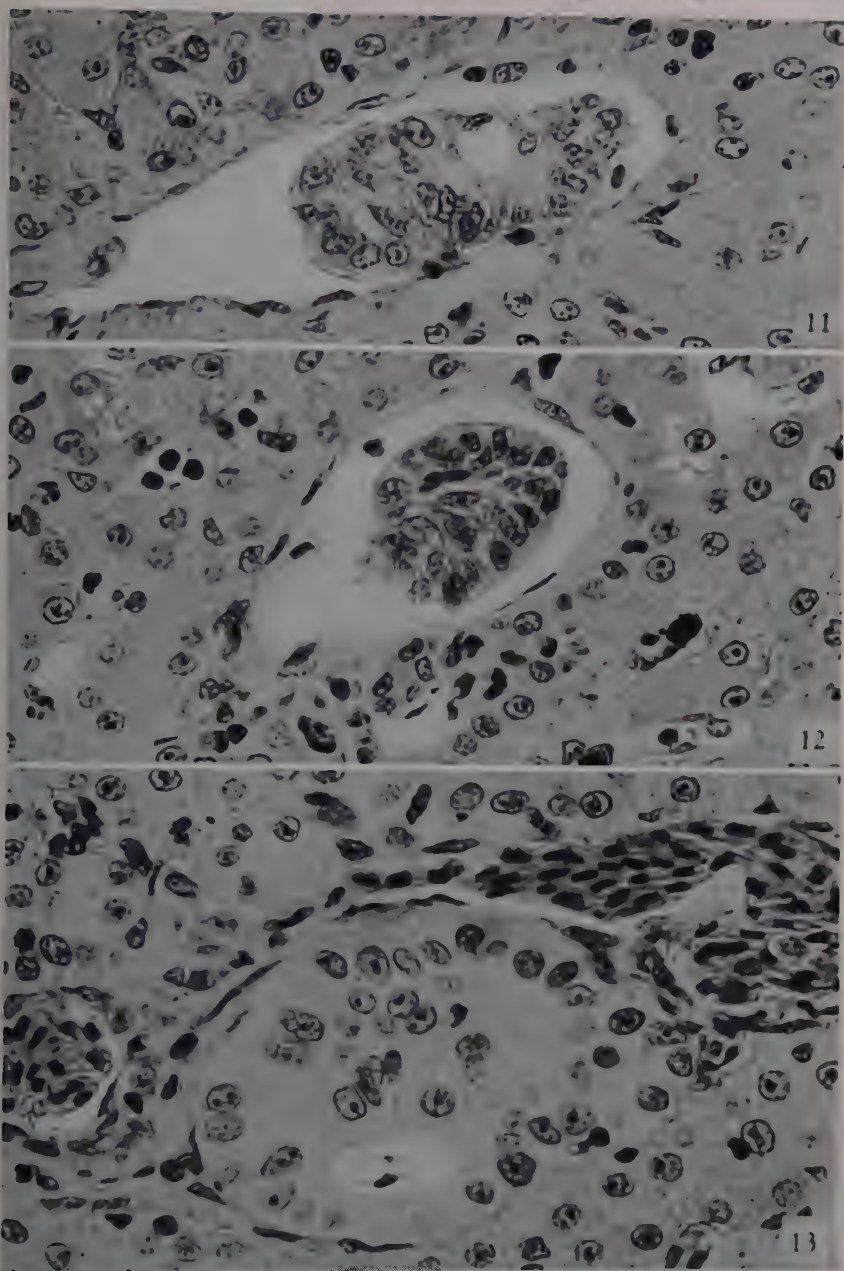


FIGURE 10. Frog with large bilateral renal tumors and numerous metastases. The latter are best seen in liver and lung, but several other organs are also involved. Magnification: 2X.

Evidence that the Frog Carcinoma Is Caused by a Virus

We now come to the evidence that the agent inducing the frog carcinoma is very probably an inclusion-forming, organ-specific virus. The first in-



FIGURES 11-13. Tumor emboli within veins of the liver. The sections are from three different frogs. Magnification: 400X.

dication of such etiologic relationship was given by the frequent presence of intranuclear inclusions in the neoplastic cells.¹ It is possible that these inclusions represent a "passenger virus" but, as stated above, they are

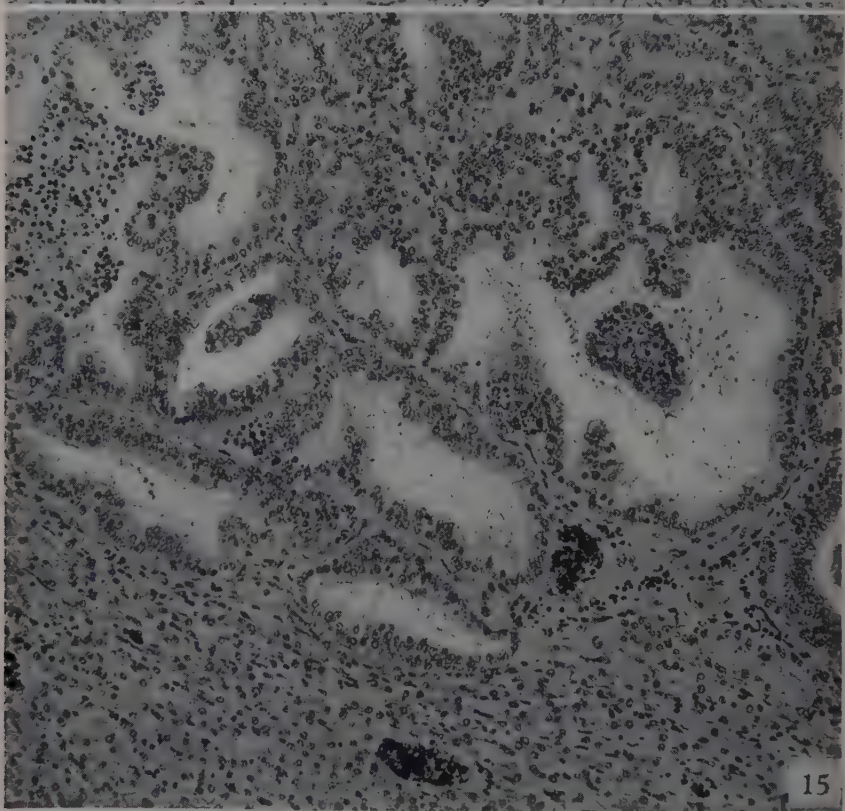
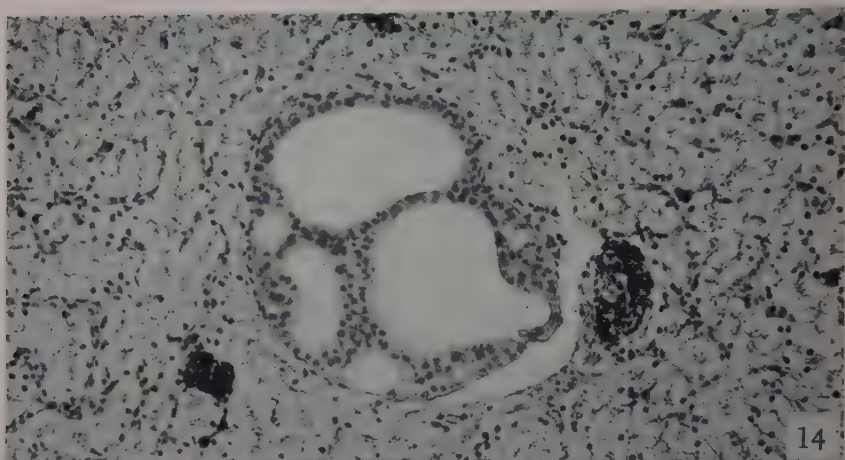


FIGURE 14. A tumor embolus within a vein of the liver. Shows an early phase of growth, as indicated by the formation of several gland-like structures. The tumor cells have not yet penetrated the wall of the vessel. Magnification: 100X.

FIGURE 15. A metastatic tumor in the liver. Magnification: 100X.

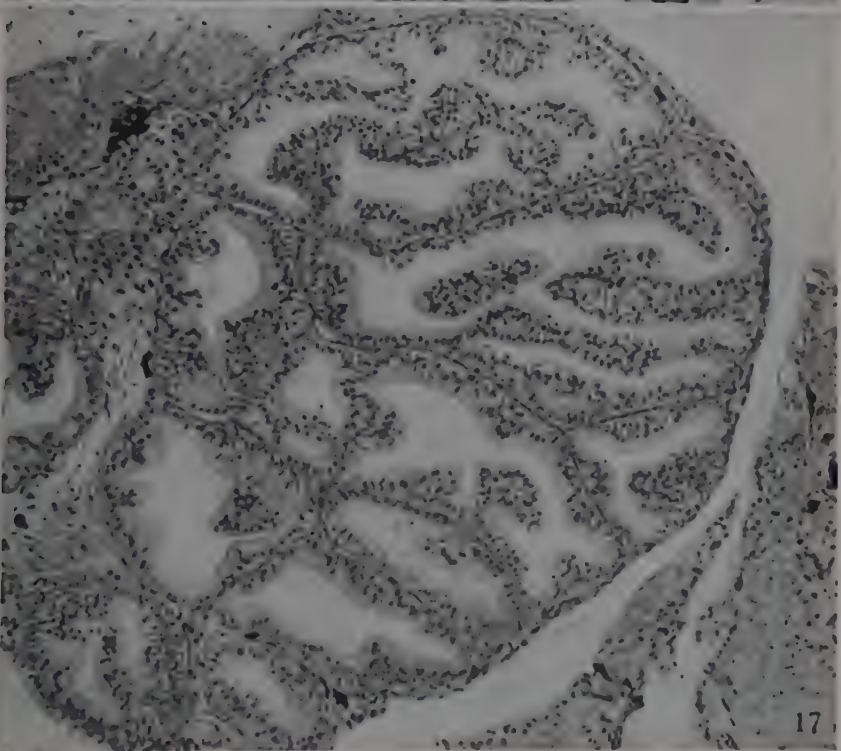
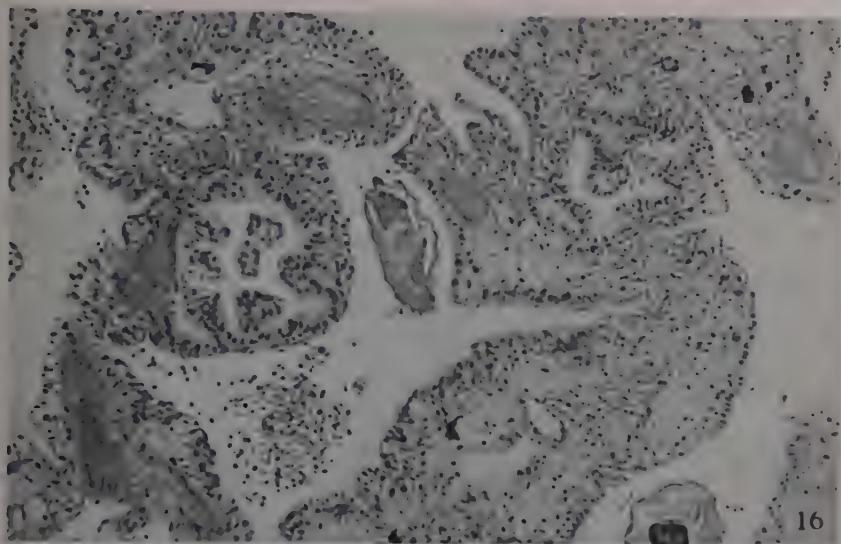


FIGURE 16. Tumor emboli within vessels of the lung. Magnification: 100X.

FIGURE 17. A metastatic tumor in the lung. Magnification: 100X.

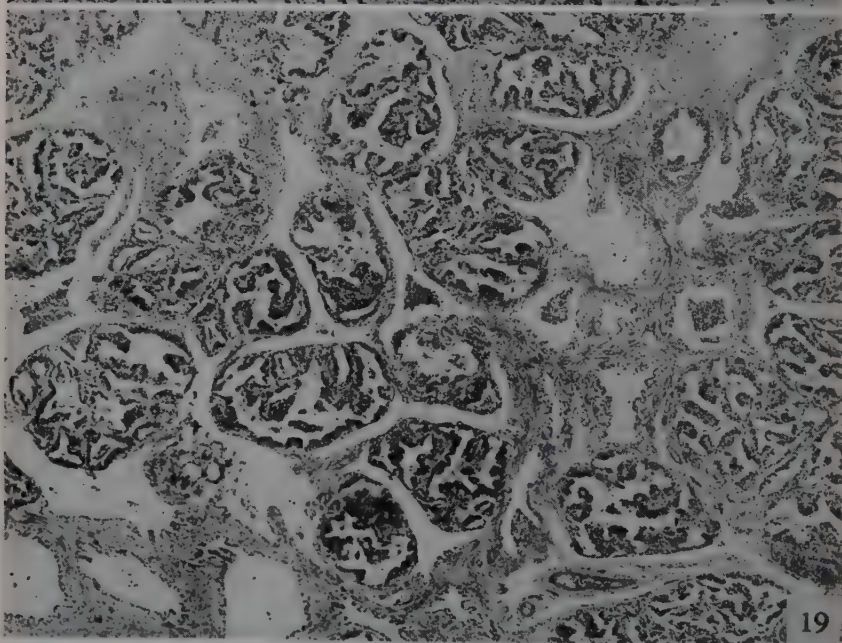
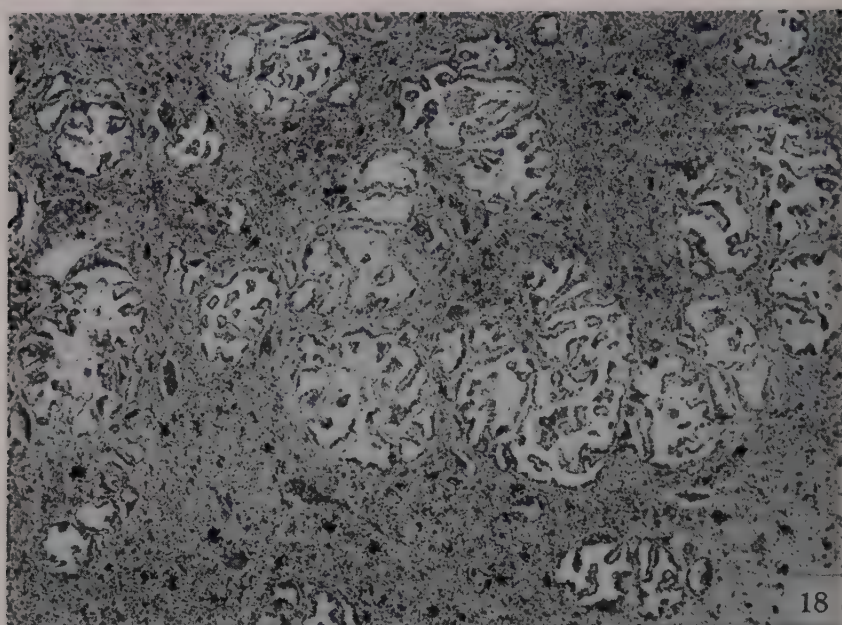


FIGURE 18. Liver riddled with numerous metastases. Shown at low magnification: 30X.

FIGURE 19. Numerous metastases in the lung. Magnification: 30X.

always confined to neoplastic cells. They never occur within the normal cells of the kidney or other organs. Even when regenerative hyperplasia of

normal renal epithelium has taken place in tumor-bearing kidneys, as often happens in consequence of damage inflicted by larval trematodes in the kidney, no inclusions are ever seen in the newly formed hyperplastic cells. The absence of inclusions from proliferating but non-neoplastic cells makes it unlikely that a "passenger virus" is concerned and supports the belief that the inclusions are indicative of a tumor-inducing virus.

The next suggestion was derived from experiments in which tumors were successfully transplanted into the anterior chamber of the eye. In frogs permitted to survive more than three months, the incidence of kidney tumors was found to be unusually high. It seems unlikely that the frequent occurrence of kidney tumors in such frogs can be attributed solely to chance. It is also improbable that the intraocular tumors had sent off emboli that became established in the kidney but in no other organ. The remaining possibility is that some agent separable from the tumor cells had become disseminated and incited tumor growth of particular cells, namely renal epithelium. A group of transmission experiments supported this supposition.

Transmission Experiments. Four series of transmission experiments were designed to test the working hypothesis that the frog tumor is caused by an organ-specific agent having the attributes of a virus.² The tumors used ranged in size from early growths to large cancers. Since it seemed possible that, in their various stages of growth, they might vary in transmissibility, it was deemed best to use a number of tumors from different frogs for inoculation, thus minimizing variable factors. The frogs were kept in groups of not more than twenty, in large glass tanks in an amphibian vivarium in which the temperature, during the course of these experiments, ranged around 45° to 50° F. in the winter, and around 65° to 70° F. in the warmer months. A total of 1026 adult frogs, in groups of 10 to 40, received inoculations from 44 different tumors. 953 frogs were maintained as controls, under the same conditions as those in the experimental series. The results of these experiments will now be reported.

Results with Living Tumor Inoculated at Various Sites. Small fragments of freshly prepared tumor cell suspensions were implanted into the muscles of the thigh, into the lymph sacs (i.e., subcutaneously), the coelomic cavity, or into the brain. The frogs were sacrificed at intervals, or examined whenever they died. As had been found in preliminary experiments, usually no significant local tumor growth resulted at any of the inoculation sites and the implanted material became resorbed. In a considerable number of frogs, however, tumors developed in the kidney and, with time, the incidence of the renal tumors increased significantly (TABLE 2). In frogs examined within the first three months after inoculation, the incidence of kidney tumors corresponded to that observed in the control groups (i.e., about two per cent). During the next three months the incidence rose in the experimental frogs and, in the frogs that survived for more than six months, tumors identical with the naturally-occurring neoplasms developed in the kidneys of more than 20 per cent of the animals. These results suggest the dissemination from inoculated tumor cells of an agent that, after a relatively long incubation period, specifically induces the

development of a kidney tumor. In this connection, it should be recalled that most vital processes of frogs are carried on more slowly than those of warm-blooded animals and also that, for several months during the course of the experiments, the frogs were kept at a low temperature.

Results with Living Tumor Implanted into the Kidney or the Liver. When small bits of cell suspensions of living tumors were surgically implanted directly into the kidneys or the liver, little or no local growth of the grafts

TABLE 2
INCIDENCE OF KIDNEY TUMORS DEVELOPING IN FROGS INOCULATED WITH LIVING RENAL TUMOR IN VARIOUS SITES

Site of inoculation	Number inoculated	Months after inoculation					
		0-3		4-6		Over 6	
Intramuscular.....	133	75	3	36	1	22	1
Subcutaneous.....	196	128	1	32	2	36	5
Intracranial.....	37	22	0	1	1	14	4
Intra-abdominal.....	200	89	0	33	5	78	17
Non-inoculated controls.....	(953)	683	16	166	10	104	7

The italic figures give the number of frogs with tumors.

It is seen that there is a rise in incidence of renal tumors after the initial 3 months' period, except in the group that had been inoculated in the muscle. Approximately 20 per cent of the other animals that had survived over 6 months had developed kidney tumors.

In the bottom line, the incidence in the control series is given. The rise in incidence is slight. Its possible significance is discussed in the text.

TABLE 3
INCIDENCE OF KIDNEY TUMORS DEVELOPING IN FROGS INOCULATED WITH LIVING TUMOR TRANSPLANTS IN KIDNEY AND LIVER

Site of inoculation	Number inoculated	Months after inoculation					
		0-3		4-6		Over 6	
Kidney.....	68	31	2	19 •	7	18	7
Liver.....	39 •	19	0	10	3	10	3
Total.....	107	50	2	29	10	28	10

The italic figures give the number of frogs with tumors.

In both experimental groups, there is a significant rise in the incidence of kidney tumors after the initial 3 months' period.

was noted in frogs examined during the first three months. During the following three months, however, kidney tumors were present in more than one-third of the frogs. As shown in TABLE 3, this high incidence was maintained in the frogs examined after a six-month period. These experiments may signify that the tumor can be transplanted successfully to the kidney, but not to the liver, or they may signify that, as in the preceding series, the tumor-inducing agent becomes disseminated and induces tumors in the kidney.

Results with Desiccated or Glycerinated Tumor. Experiments were next designed to find out whether the frog tumor can be transmitted by tumor

material containing no living cells. In view of the well-known facts that some viruses may be inactivated during filtration and that not all viruses are filterable, it was decided to use tumor desiccates (and, in one small group, glycerinated tumor) rather than filtrates. The desiccates were prepared from ten frog tumors by freezing the minced tumors at approximately -80°C . in a mixture of cellusolve and solid CO_2 , and then drying them by high vacuum distillation from a frozen state. The containers were sealed under vacuum and stored at refrigerator temperature for at least two or three weeks. The dried material was then ground to a fine powder under aseptic conditions and suspended in sterile water, 0.5 cc. of the suspension being injected.

One small group of ten frogs received an emulsion of glycerinated tumor. Slices of the tumor had been stored for 20 days in 50 per cent glycerin at refrigerator temperature. They were washed repeatedly in amphibian

TABLE 4

PERCENTAGE OF FROGS DEVELOPING KIDNEY TUMOR AFTER INTRA-ABDOMINAL INOCULATION WITH (a) DESICCATED (OR GLYCERINATED), AND (b) LIVING TUMOR MATERIAL

Tumor material	Number inoculated	Months after inoculation					
		0-3		4-6		Over 6	
Desiccated or glycerinated	244	112	6.3	38	10.5	94	21.3
Living	200	89	0	33	15.2	78	21.8
Non-inoculated controls	(953)	683	2.3	166	6.0	104	6.7

The italic figures give the percentage of frogs with tumors.

In frogs surviving more than 6 months, the results of the two experimental series are approximately alike.

Statistical analysis by the method of Brandt and Snedecor¹¹ indicates that desiccated, glycerinated and living tumor tissue are equally effective in the production of kidney tumors.

Ringer's solution, ground to an emulsion, and 0.5 cc. of the emulsion was injected.

Two hundred and thirty-four frogs received intra-abdominal inoculations of tumor desiccates, and ten frogs received emulsions of glycerinated tumor. The results of both series corresponded closely with those obtained from intra-abdominal injection of living tumor material. The incidence of kidney tumor increased with time. 21 per cent of frogs surviving the injection for more than six months developed renal tumors (TABLE 4).

It is unlikely that the tumor desiccates contained living cells. Hence, the conclusion is warranted that the tumor-inducing agent is separable from cells. These experiments, and the frequent presence of intranuclear inclusions within the neoplastic cells, make it very probable that the carcinogenic agent has the attributes of a virus.

Inoculation of Foreign Species of Frogs. Mixed desiccates of two tumors were injected into the coelomic cavity of 44 green frogs (*Rana clamitans*) or half-grown bullfrogs (*R. catesbiana*). Living material from four other tumors was introduced, also intra-abdominally, into 65 frogs of a subspecies of *R. pipiens*.

None of these frogs of foreign species or alien race developed renal tumors, which indicates that the causal agent is species specific.

Controls. Mode of Spread of Tumor in Nature

Examination of 953 control frogs gave very different results from those of the experimental series.² As shown in the bottom lines of TABLES 2 and 4, the incidence of renal tumors in the non-inoculated control groups (which, as has been stated, were kept under precisely the same conditions as the experimental groups) was about two per cent. However, with time, the incidence rose slightly, to six per cent in the second or four to six months' period and to 6.7 per cent in frogs surviving for more than six months. Although this rise in incidence is far below the increase in the experimental group, it may have much significance. The neoplastic disease may be transmissible from frog to frog by "natural" means. Captive frogs are, of necessity, kept under more crowded conditions than exist in their natural environment. Such crowding would favor transmission if, for example, the causal agent of the kidney tumor is present in urine.

To test the possibility of transmission of the tumor-inducing agent by simple contact, frogs with large palpable kidney tumors were placed in tanks with presumably normal frogs. Whenever a tumor-bearing frog died, he was replaced by another frog having a tumor, but none of the normal frogs were replaced. Fourteen such groups were maintained for over one year, each group consisting of eighteen normal and one or two tumor-bearing frogs. The results were disappointing. In a few groups, there was a distinct rise in incidence of renal tumors. In other groups, no such rise occurred.¹⁰ It, therefore, seems doubtful that the neoplastic disease is transmitted by contact with virus-containing urine or by other direct means. No further information was obtained as to the spread of the neoplastic disease in nature.

Nature of the Tumor-inducing Agent

The evidence, reviewed, points to an inclusion-forming, organ-specific virus as the most probable cause of the frog kidney tumor. The filterability, size, and various other physico-chemical attributes of the tumor-inducing agent are unknown. There is, as yet, no information as to whether continued growth of the cancer depends upon continued existence of the agent within the neoplastic cells. These and many other questions about the frog tumor require further investigation.

Summary

1. The tumor that commonly occurs in the kidney of leopard frogs is a true malignant neoplasm. It infiltrates and destroys renal tissue. It has the potentiality of metastasizing. It is serially transplantable in the anterior chamber of the eye. Finally, the activities of certain of its enzymes are altered in a manner characteristic of mammalian cancers.

2. Prominent acidophilic intranuclear inclusions are frequent in the neoplastic cells.

3. When inoculated as living fragments or as cell suspensions in various sites, no significant local growth results (except in the eye and perhaps in the kidney). In frogs surviving inoculation for more than six months, 21 per cent of the animals are found to have developed tumors in the kidney, although the inoculations were made at distant sites. These tumors are like the naturally-occurring neoplasms.

4. Desiccated or glycerinated tumor gives the same result as inoculation with living tumor.

5. In foreign species, or alien races, of frogs, no kidney tumors are induced by inoculation of either living or non-living tumors.

6. Nothing is known about the spread of the neoplastic disease under natural conditions.

7. The physical and chemical characteristics of the tumor-inducing agent have not as yet been investigated.

8. It is concluded that the kidney tumor of leopard frogs is very probably caused by an inclusion-forming organ-specific virus.

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INTERACTION OF TUMOR AGENTS AND NORMAL CELLULAR COMPONENTS IN AMPHIBIA*

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A general theory of cellular differentiation is beginning to emerge.^{4, 7, 10, 12, 15, 18-22} An essential part of the theory is that cellular differences which arise through interaction of genes and environment are stabilized in self-perpetuating cytoplasmic substances. These are known as plasmagenes and, at least in some cases, are the complex, enzyme-containing nucleoprotein-lipid particles of the cytoplasm, or are borne by them.

Some of the tumor viruses or tumor agents seem to be particles of this nature. Avian sarcoma agents are similar in composition and size to normal cytoplasmic particles and are antigenically similar.^{1, 2, 5, 6, 13} Further, tumor agents, to some extent, direct cellular differentiation.

The early work of the Rous group demonstrated that tumor agents from three histologically different tumors not only induce rapid growth but also determine specific histological characteristics.¹⁷ For example, one of them, an agent from a chondro-osteosarcoma, induced cells at its site of injection in a muscle to grow and produce an abnormal cartilage-bone tissue. The induced tissue was very much the same as the tissue from which the agent had been obtained. Murphy,¹⁴ in lectures delivered in 1935, pointed out very clearly that these tumor agents function in part as determiners of cell type. Taken as a whole, the evidence indicates great similarity between tumor agents and normal, self-perpetuating and character-determining cellular components. In the past few years, Mrs. Rose and I have been following the interaction of tumor agents and differentiated components of normal cells in *Amphibia*. The detailed data on which the following summary is based are being published elsewhere.¹⁶

Modification of the Renal Carcinoma Agent

The general method of modification has been to break down the extremely narrow specificity of the Lucké carcinoma agent by having it grow in foreign races and even in a different family. If the agent, obtained from a renal carcinoma of a *Rana pipiens* of the Lake Champlain region, is used on closely related frogs of the same region, only renal carcinomas are induced. If, however, a frog tumor is cultured in either young newts, *Triturus viridescens*, or in regenerating tissue of adult newts, it will occasionally induce abnormal growth in newt tissues.

A section of one of the original renal carcinomas of a frog used in these studies is shown in FIGURE 1. Parts of this tumor were transferred to limbs of young *Triturus*. Several abnormal growths were induced. Two began in periosteal tissue and differentiated as cartilage. One appeared as an outgrowth from the humerus and was used as a source of material for

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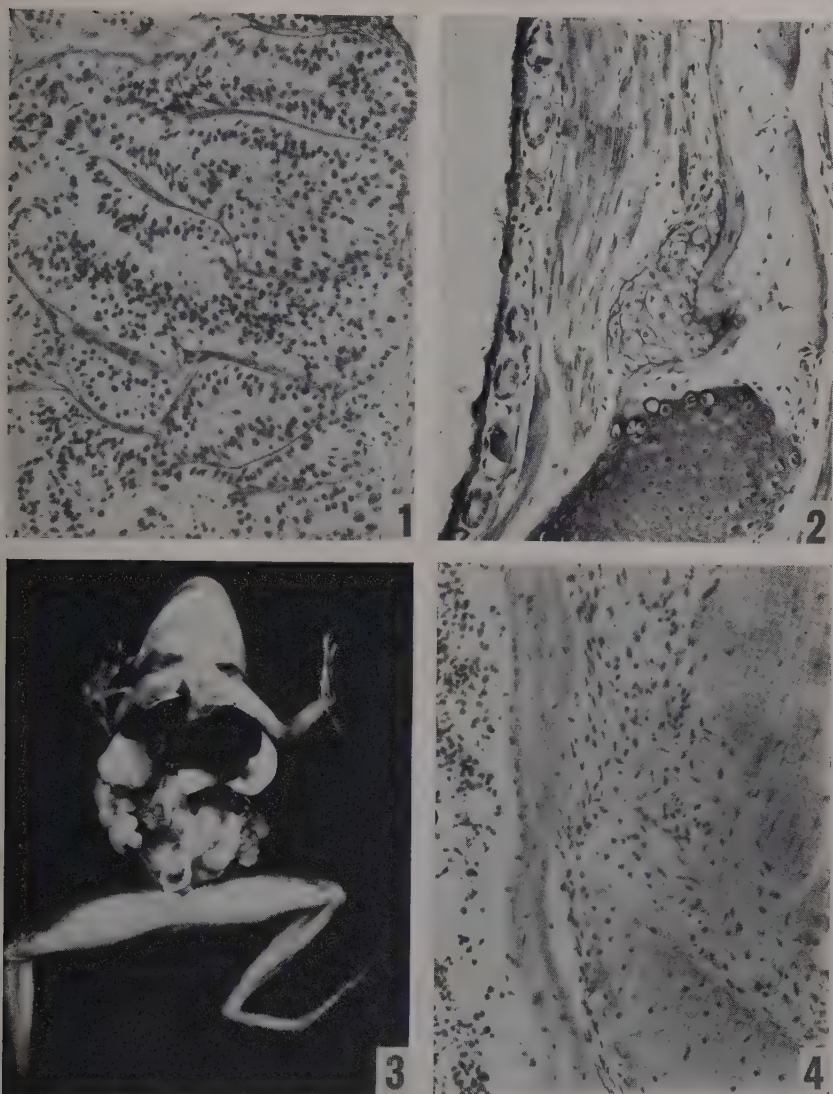


FIGURE 1. A section of RT10, the source tumor for the renal-periosteal series. Transplanted to limbs of newts. 150X.

FIGURE 2. Cartilaginous growth, CS2, induced in limb of eft stage newt by transplant of RT10. Abnormal cartilage growing in the periosteal region of distorted radius. 100X. A sister growth in the same limb was transplanted to anterior eye chambers of *R. pipiens* of Wisconsin.

FIGURE 3. RT45, a massive renal carcinoma extending as a white lumpy mass throughout the abdominal cavity. This was induced by CS2 in eye chamber. RT45 was transplanted to other eye chambers.

FIGURE 4. Periosteal growth in wrist induced by eye chamber implant of RT45. Marrow and bony shaft to left, hypertrophying periosteum in center in which cartilage is differentiating below. 150X.

further transfers. A photograph of a smaller abnormal cartilaginous growth, on the radius of the same limb, is shown in the middle of FIGURE 2. Its sister growth, also composed of *Triturus* periosteal cartilage, was transferred to anterior eye chambers of *Rana pipiens* from Wisconsin. The object

of the transfer was to learn which, if any, tissues in the frog would become neoplastic. The first tumor to appear was a typical renal carcinoma (FIGURE 3). Later, two other renal carcinomas appeared in Wisconsin frogs. This is significant, because these were the only three Wisconsin frogs used for the test. Tumors of this type are rare in Wisconsin frogs. None has arisen in over 300 controls kept under observation. Further, the test frogs were younger than those in which spontaneous tumors usually arise.

The renal tumor of FIGURE 3 was used as a source of material for transplantation to eyechambers of Wisconsin *Rana pipiens*. From this group, several tumors arising in periosteal tissue were obtained. One is shown in FIGURE 4. Normal marrow and bony shaft are seen at the left. Running down through the center is hypertrophied periosteum which grades off into differentiating cartilage.

It is interesting that in the Wisconsin frogs, two tissues were induced to become neoplastic, renal epithelium and periosteum. Renal epithelium of a frog and periosteum of a newt were former hosts to the agent.

Further transplantation of the periosteal frog tumors has led to loss of any obvious periosteal or renal characteristics of the agent. Diffuse tumors showing no periosteal type differentiation often arise at joints and spread through connective tissue and muscles, but they may arise elsewhere as well. The liver is a common site. Histolysis always follows in a region affected by the much modified renal agent. A section of histolyzing muscle is shown in FIGURE 5. The histolytic agent functions like a true disease virus, causing much more histolysis than hypertrophy. The possibility that an extraneous, disease-producing agent had been introduced is ruled out. If one had been introduced, lysis should have occurred at random. It was not at random, but in all five series followed the introduction of an agent which had been modified by sojourn in a foreign tissue. Nothing like this common histolytic reaction has ever been observed when an unmodified agent has been used. The agent induces histolysis only after growth in at least two tissues takes place.

A very similar series of tumors was obtained when a renal tumor agent from a frog became a part of chondrocytes in a regenerating limb of an adult newt. In both series, the frog renal agent, after modification in the chondrocytes of a newt, induced both renal and periosteal tumors. A further similarity is that the agent eventually became a histolytic agent in both series.

A striking phenomenon, observed eight times, was that an agent, modified by growth in a tissue other than its tissue of origin, was endowed by the new tissue with an affinity for the same tissue in the same region on the other side of the body. This was observed for the first time when a renal tumor of a frog induced an excess cartilaginous growth near the elbow in a regenerating limb of a newt. Shortly thereafter, a large cartilaginous growth appeared in the unoperated elbow on the other side of the body. This secondary cartilaginous growth induced both renal and periosteal tumors in frogs. Several times in the frogs, a primary growth at one joint of one toe was

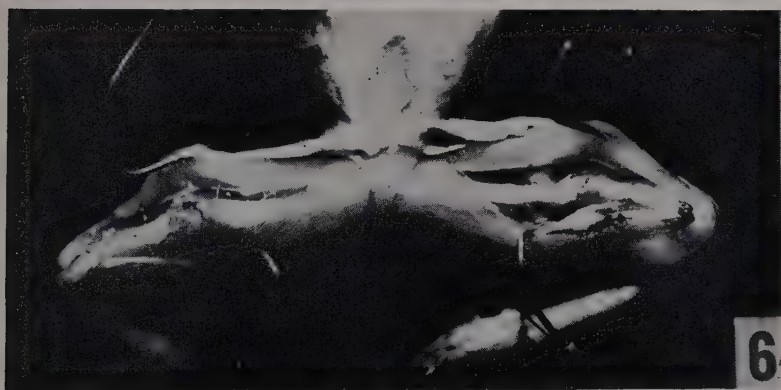
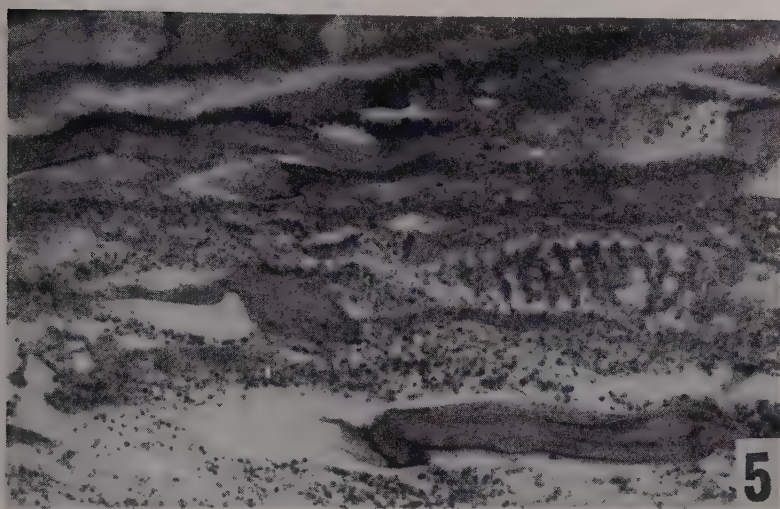


FIGURE 5. Histolyzing thigh muscle of frog which had received an eye-chamber implant of periosteal tumor shown in FIGURE 4. 150X.

FIGURE 6. Similar periosteal tumors in right and left femurs of a Minnesota frog which had received a Wisconsin renal carcinoma eye-chamber implant.

FIGURE 7. Comparison of normal fat bodies in frog on right bearing an extensive renal carcinoma with shrunken fat bodies of frog on left bearing an induced adipose tumor.

followed by a secondary growth at the same joint of the same toe on the other foot.

Bilateral tumors also arose when renal tumors were transferred to foreign races of frogs. Tissues other than renal were affected and a new agent was released which showed greater affinity for the same tissue in the exact contralateral spot than for any other tissue. In one case, for which details are given elsewhere,¹⁶ a Vermont renal agent induced rapid growth of Wisconsin iris tissue. The iris tissue in turn produced an agent which induced a tumor in a contralateral eye.

The eighth case of identical bilateral pairs occurred in the femurs of a Minnesota *R. pipiens* (FIGURE 6). The agent employed was one which had been obtained from one of the only two natural renal tumors found in over 2000 *R. pipiens* from Oshkosh, Wisconsin. It had induced typical renal carcinomas when used on other Oshkosh frogs. One of these agent-induced renal tumors had furnished the material for transplants to eyechambers of *R. pipiens* from Stillwater, Minnesota. Two out of 10 Minnesota frogs had skeletal tumors within six months. In one frog, the skeletal growth appeared in only one limb but, in the other frog, two tumors were similarly situated in the two femurs (FIGURE 6). The two larger growths are periosteal but strands from them had penetrated adjacent muscle. In addition, several apparently unconnected growths were observed in muscle and tendon. One of these is just to the right of the lower right retractor pin in the photograph. This type of spreading periosteal tissue causes extensive lysis and hemorrhage in nearby muscle. In an earlier report, such hemorrhagic lesions in muscle were thought to arise after transformation of cells in muscles. Now, after study of this early case and two others, it seems that at least some of the hemorrhagic lesions in muscle have resulted from invasion of very abnormal periosteal tissue. Similar series of tumors were followed by Duran-Reynals after modification of chicken tumor agent in other birds.^{8, 9}

An agent has been followed in three different series in its wanderings from frog kidney to periosteal tissue and back again. In a fourth series, kidney agent became a part of iris tissue. If a kidney tumor agent enters either periosteal or iris cells, what is recovered is a remodeled agent bearing the mark of its latest residence. This mark is very specific and enables the remodeled agent to enter and grow in the same tissue of the other side of the body. At the same time, an agent, after an additional transfer from the second of the bilateral sites, gives evidence of retaining its original nature. It still retains its affinity for renal tissue and its ability to induce tumors in it. The simplest interpretation seems to be that the original tumor agent or a part of it has made a new combination with a regionally differentiated normal tissue agent. The combination results in a new agent with affinity for both tissues of origin. Since tumor agents contain nucleoprotein and since other nucleoprotein or nucleic acid systems (chromosomes, various viruses including tumor agents,³ and even pneumococcal transformation agents¹¹) have been shown to make new self-perpetuating combinations, it is suggested that a combination of tumor agent nucleoprotein with normally

differentiated nucleoprotein has occurred. The implication is that the tumor agent is itself a modified normal cytoplasmic component. The supposed relationship can be diagrammatically represented (See CHART I).

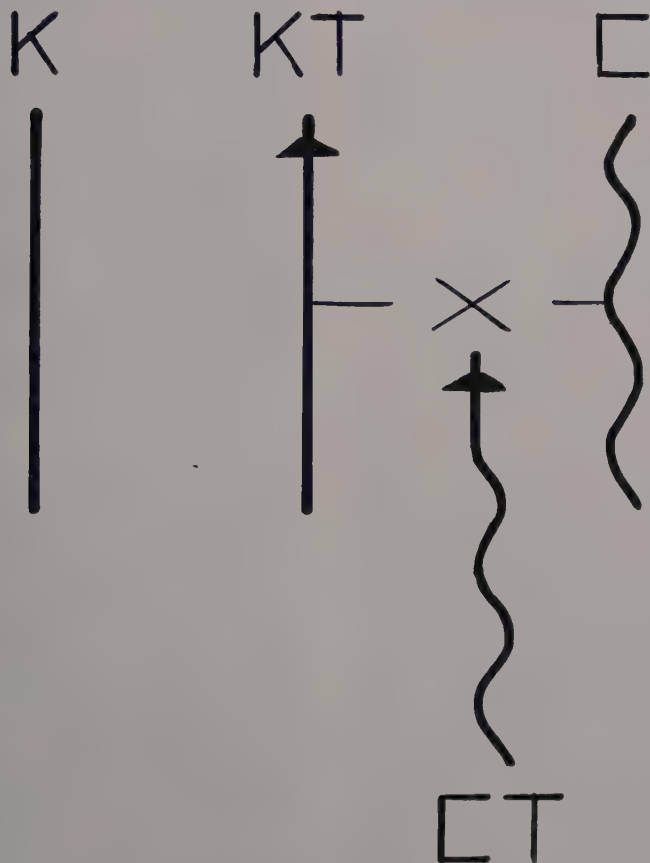


CHART I. Diagrammatic representation of the relationship of normal and neoplastic tissue agents. Agents; K—Kidney; KT—Kidney Tumor; C—Cartilage; CT—Cartilage Tumor.

Fat Body Sarcoma Series

Another natural agent tumor, discovered in a Wisconsin *Rana pipiens* by Professor Lyell Thomas, has served as a source of material for transfer studies. The original tumor (FIGURE 8) had arisen in a fat body and had directly invaded one lung. It had retained the ability of its parent tissue to collect oil. In the first of a series of transfers it was put into eyechambers of three *Rana pipiens* of Wisconsin. This tumor and the tumors derived from it always take in eyechambers (30 cases). This is in contrast to the taking percentage of natural renal carcinomas, which is approximately 30 per cent.

One of the three animals receiving the eyechamber implant exhibited

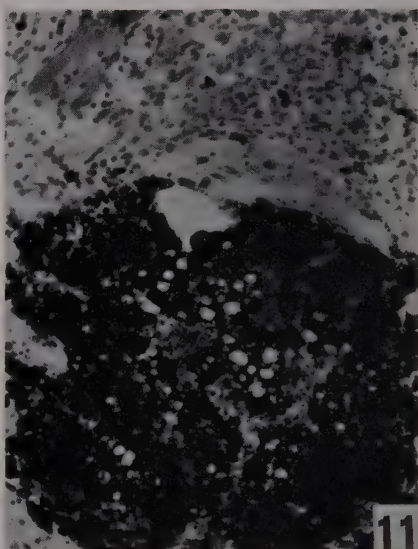
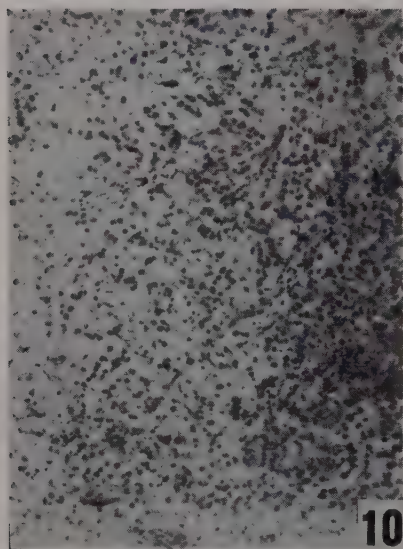
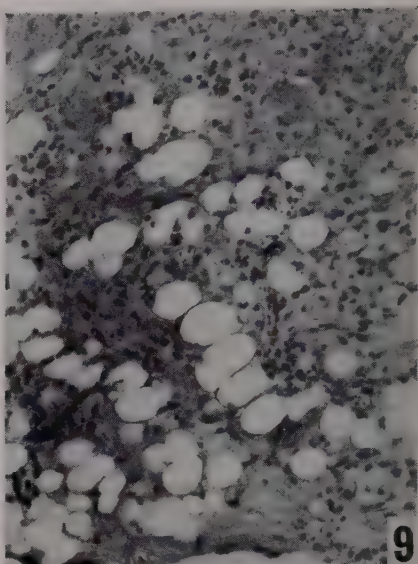
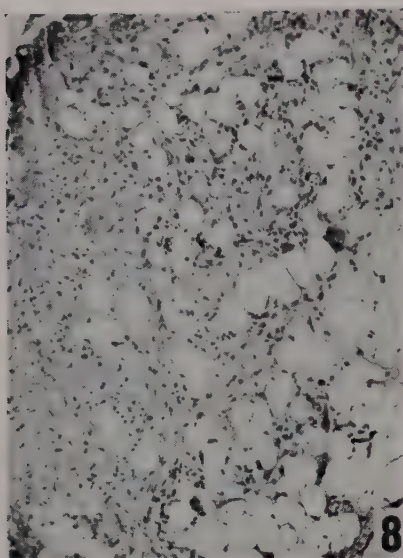


FIGURE 8. FBT1, a section of the original fat body tumor. 100X.

FIGURE 9. A section of adipose tumor of back induced by eyechamber implant of FBT1. 150X.

FIGURE 10. Small-celled sarcoma derived from adipose tumor of FIGURE 9 after passage through 2 eye chambers. 150X.

FIGURE 11. Small-celled sarcoma in ovary of frog which had received tumor of FIGURE 10. The dark mass is the remains of an egg through which the tumor had grown. 150X.

bilaterally symmetrical ulcers, 1 centimeter in diameter, on the right and left sides of the back anterior to the urostyle. The ulcers did not heal and it was noted that the exudate from them contained oil droplets. Slightly hypertrophied tissue of the body wall adjacent to the ulcers had been

modified to the adipose type (FIGURE 9). This region, which does not naturally collect and store appreciable amounts of oil, had received an agent which not only caused it to grow but also to collect oil. In fact, it became a more efficient oil repository than the normal fat bodies and caused them to diminish. FIGURE 7 shows the emaciated fat bodies of the animal in question, compared with normal fat bodies. The animal on the right also had a tumor, visible in the photograph, filling the abdominal cavity posterior to the testis, but it had a renal tumor which does not collect oil. The small fat bodies of the frog with the adipose tumor are very unusual. The laboratory diet of ground beef or horsemeat plus irradiated ergosterol and bone meal maintains large fat bodies.

The second of the three animals receiving the original fat body tumor was even more unusual. It developed multiple tumor foci: 8 in the liver, 1 in the spleen, and 1 in a mesentery. Its fat bodies had disappeared completely. The third animal lived for a year without any induced tumors appearing.

This is an interesting agent because it endows its host cells with a capacity for a special function which is greater than that of the normally differentiated cells. This refutes the often repeated and poorly founded belief that the initial change in the transformation of a normal cell to a neoplastic cell is a dedifferentiation. There is, however, no evidence of loss of special function in first generation induced tumors in either the induced adipose tumors or the periosteal tumors cited above. These tumors do not support the idea that tumor cells are more like embryonic cells than are the cells of the various normally differentiated tissues. Instead, the adipose tumor cells perform the special function of the normal tissue in such an efficient manner that they can compete successfully with the normal tissue.

During tumor transplantation there is selection for more rapidly growing and more abnormal cells. In this series, as in the periosteal series, a very malignant tissue is obtained which does not perform the special function of its parent tissue; but it would require great distortion of the imagination to consider these even more abnormal cells as embryonic cells. In the first place, they do not look like embryonic cells and, in the second, embryonic cells would not continue to grow in the situations where the tumor cells do.

Much more rapidly growing, non-adipose tissue was obtained after transfer of the first induced adipose tumor (the one which had appeared in the back). This dorsal adipose tumor was transplanted to five Wisconsin eyechambers. It grew in all five and completely destroyed the eye but, in the course of its growth, it lost its ability to store oil. Further transplantation of the growths from these eyes has not led to the production of any oil-collecting cells, either in eyechambers or in agent induced tumors elsewhere. This indicates the change in cell type is paralleled by a change in agent type. A section of one of the third transfer eyechamber tumors is shown in FIGURE 10. Its nature is now quite different from that of the original tumor (FIGURE 8) and the first induced tumor (FIGURE 9). The original tumor, although it had invaded a lung, was not highly malignant and did not destroy adjacent eye tissues when transplanted to anterior eye

chambers. The present modified tumor always destroys eyes when implanted in them. All normal tissues may be dissolved including the lens and the sclera. The tumor makes its way out of the eyechamber through the cornea or sclera and may make its way to the surface of the body or into the mouth. After transplantation to eyechambers, other tumors of the same type appear in other parts of the body, usually in liver and mesenteries, but in other regions as well. A portion of a section of one which appeared as a compact tumor 12 mm. in diameter in an ovary is shown in FIGURE 11. It is a small-celled sarcoma covering the field of the photograph. In the lower part of the photograph, a dark egg traversed by tongues of tumor tissue may be seen.

In the fat-body series as in the renal periosteal series, a rapidly growing tissue, which had lost its former differentiated characteristics, was eventually obtained.

Conclusions

Both types of tumors, the renal and the fat-body, contain agents which can be remodeled during growth in foreign tissues. While, in some cases retaining affinity for their original tissue, they acquire from the foreign tissue a very specific affinity for that tissue. This affinity sometimes is limited to a particular joint of one toe. It is suggested that a new particle arises composed of two parts, one from the tumor agent and the other from a normally differentiated particle. These combination agents are quite unstable and cannot be maintained. Instead, unless they re-enter the tissue of origin, they change to a type inducing more unusual and more rapidly growing cells. Finally, they become cytolytic agents resembling some of the more virulent disease viruses in their action.

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RABBIT PAPILLOMAS AND THE RABBIT PAPILLOMA VIRUS; A REVIEW

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Considerable evidence indicates that viruses cause certain papillomas that are frequently observed in man, cattle, dogs and rabbits.¹⁻⁴ There are certain similarities among the various papilloma-producing viruses and the induced papillomas. Taking into account the difference of the hosts, the gross and microscopic appearance of the papillomas closely resemble each other. In general, the pathogenicity of a given papilloma virus is cell and species specific. It is also characteristic for the virus-induced papillomas to undergo spontaneous regression and to disappear in a period of months. Chronic irritation or other repeated trauma seems to stimulate papillomas to a greater size and a longer duration but naturally occurring warts ordinarily have no serious sequelae except for the carcinomas that occasionally develop in the venereal warts of man, and that occur in the papillomas of cottontail rabbits. As far as it goes, the available evidence reveals no immunologic relationship between the viruses of rabbit papilloma and the viruses that cause verruca vulgaris in man, oral papillomatosis of dogs, and cattle warts.⁵

Natural History of Infection with Rabbit Papilloma Virus. In certain regions of the United States, notably Kansas and Iowa, many cottontail rabbits have been observed to bear papillomas. The growths are frequently multiple and seem to be concentrated on the thighs, abdomen, neck and shoulders, although they also have been observed in other areas of the body. Most often, the natural growths are grey-black, horn-shaped structures whose bases are rather loosely attached to the skin. The actual incidence of infection with papilloma virus is unknown, but it is recognized that some cottontail rabbits without papillomas are resistant to infection with papilloma virus. Presumably, this resistance is due to immunity caused by papillomas that had regressed or had been removed by trauma. Laboratory observation of naturally and experimentally infected rabbits has revealed that similar factors influence the course of the virus-induced papilloma in both cottontail and domestic rabbits.⁷ The speed and magnitude of the resulting growth closely corresponds with the potency of the infecting virus. Certain indefinable attributes of the skin appear to influence the character of the resulting papilloma. For example, skins that react vigorously to injections of Schlarlach R and certain other irritants usually give rise to large papillomas. Local conditions also affect the course of the papilloma once it has arisen. Repeated trauma, effected by various natural and artificial means, is generally associated with more vigorous growth of longer duration.

Considerable attention has been directed to the problem of papilloma regression. In cottontail rabbits, the tumors grow less vigorously and regression occurs more often and earlier than in the domestic rabbit. The actual reason for this difference in species response is unknown although it

is commonly postulated that it is referable to some host-parasite relationship which has developed in the natural host, the cottontail. Several likely reasons for regression have been studied. It has been established that papilloma antibodies that develop in infected animals do not cause the regression. Large papillomas of long duration commonly occur in rabbits that have potent papilloma neutralizing antibodies in their sera.⁶ Inside the papilloma cell, the virus is ordinarily well protected from antibody. Other studies have revealed that disappearance of the virus from the papilloma is not the reason for regression. Indeed, virus of high potency has been isolated from cottontail growths that have undergone marked regression.⁶ There is reason to believe that some little understood host factor is of great importance in effecting recession of the papillomas. It has been observed repeatedly that all growths of a given animal usually regress at essentially the same time. Furthermore, in rabbits bearing growths resulting from simultaneous infections with viruses of high and low potencies, the papillomas also generally recede at the same time without regard to the potency of the initiating virus.

The natural mechanism of transfer of the virus from rabbit to rabbit is not known. Although little papilloma virus can be demonstrated in the keratinized upper portion of the tumor, it is possible that papillomas are spread by traumatic contact between rabbits. Because papillomas are not commonly observed on the lower legs it seems unlikely that indirect spread through contact with the virus contaminated environment of the rabbits is an important means of virus transfer. In Minnesota, where the warts have been observed to be predominantly located about the head and neck, it has been reported that rabbit ticks (*Haemaphysalis leporis-palustris*) that were allowed to feed on a papillomatous cottontail ear transferred the virus to another cottontail rabbit.⁸ Natural infection with the rabbit papilloma virus seems virtually confined to the cottontail rabbit. If rabbit ticks are natural vectors of rabbit papilloma virus in the cottontail, one wonders why they do not also transmit papillomas to their most common host, the snowshoe hare.^{9, 10}

The Virus and its Characteristics. The physical characteristics of rabbit papilloma virus are well defined. Papilloma virus is one of the hardy viruses. Heating at 63° to 67°C. for one-half hour is necessary to inactivate it.^{4, 11} Papillomas stored in glycerine-saline for as long as 20 years still retain considerable active virus.¹² The results of studies employing ultracentrifugation, filtration and electron microscopy indicate that the virus is a spherical particle with a diameter of approximately 50 mμ.¹³⁻¹⁵ A relatively pure protein has been obtained from extracts of cottontail rabbit papillomas by differential centrifugation. It is believed that this protein represents papilloma virus because its concentration was found to parallel the infectivity and the complement-fixing antigen of the tumor extracts. It is significant that this protein could not be isolated from large quantities of domestic rabbit papilloma extract.^{16, 17}

Papilloma antigen-antibody reactions have been carefully studied. It is agreed that papilloma virus has no soluble antigen and that neutralization, complement fixation, flocculation and adsorption reactions are all dependent

on the presence of the papilloma virus.^{11, 18} Likewise, it has been demonstrated that the various antigen-antibody reactions are all dependent on the same specific papilloma antibody.¹⁹ For these reasons, and because it gives quick, easily reproducible results with the papilloma system, the complement fixation test has been employed wherever practical for the demonstration of papilloma antigen and antibody.

Pathogenesis of Infection. Experimentally, infection is usually initiated by introducing virus into the skin of the abdomen by tattooing or sacrifice. The former method gives rise to discrete growths that resemble the natural tumor, whereas papillomas arising in areas of scarified skin become large cauliflower-like tumors. Studies of histologic sections taken at intervals after scarification inoculation revealed that almost all the epidermal cells were removed in scarification and that the virus apparently exerted its effect on the actively proliferating cells that extend to cover the denuded area.²⁰ Macroscopic evidence of papilloma can be noted eight to twenty or more days after infection is initiated. Once the papillomas appear in cottontail rabbits, virus usually can be detected in large quantities until the tumor disappears. It should be noted, however, that virus cannot be detected in all cottontail papillomas and that experimentally produced cottontail tumors contain less virus than natural ones.²¹

In domestic rabbits the situation is radically different. When the virus etiology of rabbit papillomas was first investigated, a major stumbling block was the inability to recover virus from domestic rabbit tumors, despite the fact that the rabbits developed neutralizing and complement fixation antibodies for the papilloma virus and were relatively resistant to re-infection.⁴ In certain instances, however, it was possible to demonstrate small amounts of virus and, on at least two occasions, ten serial passages of papilloma virus in domestic rabbits were effected.^{22, 23} The antigenicity of domestic rabbit papillomas was also demonstrated by inoculating extracts of the tumors intraperitoneally into normal rabbits. Some of the rabbits developed complement-fixing and neutralizing antibodies and an even larger number, on challenge, showed resistance to re-infection.^{24, 25} Investigation of the masked antigen responsible for the development of specific antibodies in the intraperitoneally inoculated rabbits revealed that the antigenicity was at least partly due to the presence of papilloma virus not effective in producing papilloma by ordinary inoculation techniques. These smaller amounts of virus were demonstrated by inoculation of extracts of papilloma into skin made hyperplastic before scarification and covered with paraffin gauze after incision. The very early epithelization of the wound that was not accompanied by necrosis appeared responsible for the demonstration of the virus.²⁶ The total amount of virus recovered by these refined techniques however, was usually a small fraction of that demonstrable in equivalent amounts of cottontail papillomas. Other investigations revealed that the serum antibody sometimes masked the presence of papilloma virus by diffusing into papilloma tissue and combining with virus.^{21, 27} There still remained some domestic rabbit tumors (frequently vigorously growing ones) in which no virus could be demonstrated by any technique and that contained little or no papilloma antibody.²⁷ Satisfactory reasons have been

sought to explain why rabbits bearing these particular tumors developed papilloma antibodies. The most obvious possibility is that papilloma virus was present in such small quantities that it could not be detected by the available techniques for determining infectivity. It also seemed possible that virus was present but inhibited by some unknown substance, or else the virus was present in some non-infective form that retained its antigenic capabilities. Efforts have been made to demonstrate an inhibitory substance in domestic rabbit papillomas. Advantage was taken of the relative heat stability of the papilloma virus to try to destroy papilloma inhibitors at temperatures that would not inactivate the virus. These attempts were not successful in revealing the presence of virus inhibitors.²⁸ The problem has been approached in another way by incubating papilloma tissue with papilloma virus. It was reported that domestic rabbit but not cottontail rabbit papilloma tissue appeared to hydrolyze cottontail rabbit virus.²⁹ The significance of this finding however, is not clear because it was impossible to test adequately the effect of normal domestic rabbit epidermal cells on cottontail papilloma virus. Furthermore, the effect of other domestic rabbit tissues on cottontail papilloma virus was not reported.

Papilloma Virus Variation. The development of squamous cell carcinoma in many domestic rabbit papillomas and in some cottontail rabbit papillomas stimulated workers to try to obtain evidence that a papilloma virus variant was the cause of the transition from papilloma to carcinoma. Because papilloma virus has never been recovered from carcinomatous tissue derived from rabbit papilloma, other evidence of papilloma virus variation has been sought. Thus, it has been demonstrated that papilloma virus obtained from certain cottontail rabbits causes tumors in the domestic rabbit from which small amounts of active papilloma virus can be regularly demonstrated, while other cottontail papilloma viruses cause tumors in the domestic rabbit in which active virus can rarely if ever be demonstrated.²⁶ This at least suggests certain strain variations. Nevertheless, the attempts that have been made to induce variation in papilloma virus by treating papillomas *in vivo* with X-rays and methylcholanthrene and by methylcholanthrene treatment of papilloma virus *in vitro* have been unsuccessful.³⁰ The effect of papilloma virus on tar-induced tumors and the effect of chemical carcinogens on virus-induced papillomas have been carefully studied for evidence of papilloma virus variation. Repeated applications of tar causes the development of papillomas that closely resemble the virus-induced papilloma. The tar tumors, themselves, however, rarely become squamous cell carcinomas. When papilloma virus is injected intravenously into rabbits whose ears have been tarred for several months, dramatic changes occur in the ears. Virus papillomas appear, tar papillomas increase in size, carcinomatoid tar tumors sometimes rapidly become frank squamous cell carcinomas and in some instances hybrid tumors develop.^{31, 32} The incubation *in vitro* of papilloma virus and minced tar tumor and the subsequent injection of the fragments of tissue subcutaneously and intramuscularly into the homologous rabbit gives rise to similar phenomena.³³ Prolonged application of chemical carcinogens, especially methylcholanthrene, to virus-induced papillomas brought about the development of many carcinomas

within 60 to 80 days of papilloma infection.³⁴ Because these experiments were performed with domestic rabbits, efforts were not made to recover and test the papilloma virus for any induced variation. Similar experiments were therefore undertaken utilizing papilloma virus and tar-induced carcinoma tissue from the cottontail rabbit ear (where tar easily induced squamous cell carcinoma). Here the papilloma virus at times seemed to affect the carcinoma tissue so that, histologically, it appeared more invasive and anaplastic. When, however, virus was recovered in small amounts from the transplants (that may have contained papilloma tissue as well as cancer cells), it proved to be unaltered papilloma virus.³³ The evidence at hand seems to indicate that the ability of papilloma virus to modify tumor tissue as well as scarified normal rabbit skin is one of the intrinsic qualities of the virus.

Summary. The rabbit papilloma virus is one of several viruses that has the ability to produce more or less self-limited tumors of the epidermal tissue. In its natural host, the cottontail rabbit, papilloma virus is ordinarily easily recovered from the warts it produces. Papillomas induced by the cottontail virus in the domestic rabbit are of special interest because relatively small amounts of virus, or none at all, can be recovered and because squamous cell carcinoma develops so frequently in the papilloma. Neutralization of papilloma virus by specific antibody significantly decreases the amount of virus that can be demonstrated in the domestic rabbit papillomas. Other factors, however, at present undefined, are also believed to mask the presence of the virus. No definite evidence has been produced, as yet, to support the attractive hypothesis that papilloma virus variation plays a role in the development of carcinoma in the rabbit papilloma.

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THE PATHOGENESIS OF THE RABBIT PAPILLOMA-TO-CARCINOMA SEQUENCE*

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A naturally-occurring cutaneous papilloma is commonly present on wild hares captured in states bordering the Mississippi River.^{25, 40, 41} This epithelial tumor became interesting to many investigators following the discovery in 1933 by Shope⁴¹ of its virus etiology and the demonstration by Rous and Beard^{33, 34} and others^{15, 24, 48} that the benign virus-induced growth may terminate in an epidermoid carcinoma. A wide variety of investigations of this tumor and its etiological agent resulted. Our interest in this disease and in the causative virus has been continuous since June, 1934.⁴⁶⁻⁵⁴ Since then, we have kept in our rabbit colony more than 1700 wild cottontail and domestic rabbits with papillomatosis. Our cumulative data and comparative studies^{51, 52, 54} reflect a wide experience that has confirmed and extended work previously reported. It is the purpose of the present paper to summarize these findings§ and the reported experiences of other investigators in an attempt to understand two phenomena of fundamental importance: (a) the role of Shope's papilloma virus in the papilloma-to-carcinoma sequence and (b) the biological phenomenon that results during the virus papilloma-to-carcinoma sequence in the disappearance, or "masking," of the causative virus in papillomas of domestic rabbits and in cancers of domestic rabbits and wild hares.

The Growth Pattern

To make clear what is to follow, let me remind you of the growth pattern of papillomatosis in wild cottontail and domestic rabbits. Earlier published studies from several laboratories included accounts of the clinical and pathological findings of the virus-induced rabbit papilloma and of the cancer that may follow.^{1, 15, 16, 22, 24, 34, 41, 46} These findings made it known that the manifestations of infection by the papilloma virus were commonly predictable to eventuate in regression and recovery or in a squamous cell cancer with metastases. The differences in host response led to emphasis being placed upon the development of cancer in the domestic rabbit and upon recovery when the wild cottontail was infected. Usually, the natural sequence of changes was interrupted by experimental procedures which

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‡ The earlier portion of the work was completed while the author was a member of the Department of Bacteriology and Immunology, University of Rochester School of Medicine and Dentistry, and of the Department of Microbiology, Louisiana State University School of Medicine, New Orleans.

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were undertaken to elucidate some point at question^{11, 33, 35} or to accelerate the papilloma-to-carcinoma sequence.^{11, 33, 35, 36} However desirable such purposeful interference may be, it must modify to some extent the alterations that occur in the absence of such procedures. In the beginning therefore, we began to set aside animals from the wild cottontail and domestic rabbits with papillomatosis that were being kept in our colony for continuous observation. These rabbits fell into three groups:

Group I consisted of 207 wild, Western cottontail rabbits (*Sylvilagus floridanus alacer* Bangs) which had naturally-occurring papillomas when captured.

Group II was made up of 94 wild cottontail rabbits with experimentally induced papillomatosis. These animals, for the most part, were cottontail rabbits (*Sylvilagus floridanus mearnsi* Allen), captured in northwestern New York State.

Group III was comprised of 293 domestic rabbits (Genus *Oryctolagus*) mostly of pure-bred American Dutch strain, with experimentally induced papillomatosis. The handling of the rabbits was purposely limited to a weekly examination. Forty-four strains of papilloma virus were employed for transmission studies. These 44 strains were essentially similar in pathogenicity, except for differences attributable to the infectious titers, which ranged from 10^1 to 10^6 . Cross-hatch scarification limited to an area of epidermis 2.0 cm. in diameter was employed for inoculation. The growth pattern of papillomatosis was predictable and fell into three phases: proliferative, stationary and involutionary.

The proliferative phase was characterized by multiplication at maximal rate of the infected epithelial cells. It became evident within from seven to 42 days after contact of the injured epidermis with virus by the appearance of tiny vesiculoid papules. These papules differentiated within from a few days to several weeks into recognizable *stratum granulosum*, *stratum lucidum*, *stratum corneum*, and *stratum disjunctum*. The resultant growths ranged in size from small mounds a few mm. in height to enormous verrucous masses and cutaneous horns. To cite an extraordinary example, one horn measured more than six inches in length. These lesions were discrete on first appearance, but commonly reflected the amount of virus in the inoculum by merging in from seven to 28 days to form heaped-up conglomerate lesions, or superimposed and partially-separable single papillomas. At least three determinant factors conditioned the host response:

(1) The number of virus entities, as measured by the infectious titer, was found to determine: (a) the incubation period; (b) the immediate extent of epidermal hyperplasia; (c) the time required for the lesions to become confluent; and (d) the ultimate size.

For example, inoculation with successive tenfold dilutions of virus elicited lesions that fell into a graded series ranging from massive confluent growths produced by the 10^1 dilution, to one or two small discrete papillomas where the inoculation of a higher dilution led to the end-point. Moreover, when multiple rabbits, wild and domestic, in response to successive dilutions of a given virus suspension, each yielded a graded series of lesions, this identity

The stationary phase bridged the proliferative and involutionary phases. Grossly, the lesions commonly appeared quiescent without evidence for desiccative or degenerate changes which characterize the involutionary phase. Usually, virus was not recoverable from cottontail papillomas during this phase.

* Exceptionally and, quite unpredictably among several rabbits tested, a single rabbit reacted to infection by showing a prolongation in incubation period, a decreased tissue proliferation, and early involutionary changes with disappearance of the lesion. When an occasional domestic rabbit or cottontail rabbit captured in New York showed such evidence of resistance to infection, natural neutralizing antibody was sought but in no instance found. On the other hand, cottontails imported as normal from Kansas commonly were found to have a residual immunity, which was readily established as resulting from the presence of specific antibodies for Shope's virus. This finding has led us to be skeptical of any interpretation founded on alterations that occur in laboratory-infected Western cottontail rabbits. Accordingly, it should be recognized that the generalizations presented herein as representative of the virus-induced rabbit papilloma-to-carcinoma sequence depicts the modal response in cottontail rabbits captured in western New York State.

rabbits are allocated by serial letter: Series A—the cottontail rabbits that had papillomas when they arrived from Kansas; Series B—cottontails captured in New York or Kansas and infected experimentally; Series C—domestic rabbits infected experimentally.

It was indeed rare for papillomas to continue for more than 18 months as benign growths. The critical period for change was found to occur in the natural infections of cottontails and for the experimental infections of domestic rabbits at about the ninth month and for the experimental infections of cottontail rabbits at about the twelfth month. Since the naturally infected Western cottontails had well developed papillomas when they came under observation, it seems probable that the reactivity of all cottontails is the same and that benign lesions in this host persisted longer than in domestic rabbits.

The incidence by month of spontaneous involutionary regression of the disease is made known in TABLE 1. It can be seen that from six to 21 per

TABLE 2
PAPILLOMA-TO-CARCINOMA SEQUENCE IN 167 RABBITS OBSERVED FOR 6 MONTHS OR LONGER

Series	Host rabbit	Condition of infection	Total number	Benign		Complete retrogression		Carcinomatous degeneration	
				Num-ber	Per cent	Num-ber	Per cent	Num-ber	Per cent
A	Cottontail	Natural	112	46	41	40	36	26	23
B	Cottontail	Experimental	25	19	76	0	0	6	24
C	Domestic	Experimental	30	8	27	0	0	22	73

cent of the lesions disappeared within the first six months of observation.* After the sixth month, whereas more than a third of the naturally induced lesions disappeared, none of the experimentally induced lesions in either variety of host underwent regression. This difference was not related either to the mode of infection or to the host species, since about 25 per cent of the cottontails, whether naturally or experimentally infected, developed cancer when kept more than six months. Alterations of the natural growth pattern to result within the first six months of observation in malignancy and death of the host occurred in none of the domestic rabbits and in a single naturally infected cottontail rabbit. On the other hand, the findings (TABLE 2) for the 167 rabbits that were kept under observation for six months or longer to learn the natural papilloma-to-carcinoma sequence made it known that 25 per cent of the wild cottontails and 75 per cent of the domestic rabbits had lesions that terminated in cancer.†

* Care was exercised throughout these studies to note, on weekly inspection, the unexpected disappearance of papillomas and other evidence that lesions had been torn off inadvertently and, thereby, to avoid a false interpretation of regression. This precaution was necessary, since papillomas are readily lost from cottontails because of a tenuous and superficial attachment in contrast to their firm embedment in the dermis of domestic rabbits.

† Papillomas induced by infection of foetal rabbits *in utero*⁸ were replaced within 7 months by epidermoid carcinomas. The papilloma-to-carcinoma sequence occurred several months sooner than it had in adult animals kept without experimental interference under similar conditions.⁵¹

Infectious and Masked Variants of Papilloma Virus

The foregoing results and the earlier studies to which reference was made, make it possible to relate in summary our understanding of the role of the papilloma virus in the rabbit papilloma-to-carcinoma sequence.

Virus obtained from naturally-occurring papillomas is transmissible to both wild and domestic rabbits. The papillomas induced in experimentally infected wild hares yield virus which is readily maintained when transferred in wild cottontails indigenous to areas where the disease is not enzootic. The resultant growths are vigorous, abundant, and lasting. Infectious virus commonly persists for months in papillomas of this host species. On the other hand, it may disappear quite unpredictably at any time during the stationary, regressive, or carcinomatous phases. An occasional cottontail moreover, develops papillomatous growths that fail to yield infectious virus. This unusual occurrence in the natural host species is an expected eventuality on transfer to the domestic rabbit, for infectious virus is not recoverable from papillomas induced in this host.*

The finding that cottontail virus, on sojourn in a related but hetero-specific host, the domestic rabbit, should be altered to resist transmission either to that heterospecific host, or to the homospecific host, the cottontail rabbit, presents for consideration a phenomenon of fundamental biological significance. The infectious and pathogenic virus is transformed to an occult, "masked," non-communicable and, presumably, nonpathogenic variant. Nevertheless, the immunological specificity of the masked variant is not significantly altered, for it is antigenic on transfer to new hosts. For example, the conversion abruptly from a tumor of viral etiology to what appears to be a nonviral tumor is accomplished simply by transferring infectious cottontail virus to the scarified domestic rabbit skin. The resultant tumor, which resembles closely a cottontail papilloma and satisfies the properties of a benign transplantable neoplasm,^{1, 32} resists transmission to domestic rabbits and, surprisingly, to cottontails. If cottontail papilloma virus were not available as a reagent to establish immunologic evidence for the presence of virus, the growth would inevitably fall into the category of a nonviral mammalian tumor, even though its viral etiology might be suspect. Accordingly, the presence of the masked virus in a domestic rabbit papilloma or carcinoma must be established by employing cottontail papilloma virus to test the domestic rabbit host for active immunity or for specific anti-

* A noteworthy exception to this dictum is the experience of Shope,⁴² who effected successfully a second serial passage in 13 of 58 attempts to initiate papillomas in domestic rabbits. Four of these serial passages were maintained for 5 transfers and, in one series, 14 successive transfer passages were made. These findings were confirmed and extended by Selbie, Robinson, and Shope⁴³ and by Selbie and Robinson.^{37, 38} These investigators, upon employing a single sample of virus to initiate papillomas in cottontail and domestic rabbits, reported the successful maintenance in each host species of infectious virus for 14 successive transfers. The papillomas produced by the transmissible infection in domestic rabbits resembled closely the infectious papillomas of cottontail rabbits except that the infectivity of the transmissible domestic rabbit strain was lower than that maintained in parallel in cottontail rabbits, as evidenced by low and irregular yields of papillomas. The papillomas on rabbits that survived more than 12 months regularly were supplanted by malignant growths. The extent of adaptation to the domestic rabbit was of particular interest. For example, transfer to the cottontail of the domestic-rabbit adapted virus resulted in papillomas and, unexpectedly, in loss of the transmissible strain. The adapted virus by return to the natural host was altered to the "masked" or occult variant, as commonly occurs when cottontail virus is employed for infection of domestic rabbits. Moreover, virus representative of cottontail Passage 13 behaved as field virus derived from natural cottontail infection, for it initiated, on transfer to domestic rabbits, vigorous and abundant growth of papillomatous tissue which was not transmissible either to domestic or cottontail rabbits. This reciprocal reversion to an inapparent virus infection has wide implications in any approach to an understanding of the etiology of spontaneous, noninfectious mammalian tumors.⁴³

bodies, by assessing serum samples by the neutralization and complement fixation techniques. Other methods for the demonstration of the antigenic but noninfectious, occult, or "masked" papilloma virus are not available.

Shope⁴³ points out that virus-masking after the manner of the papilloma agent might explain the apparent absence of virus in other tumors. This possibility has received experimental support from the results of studies^{12, 19, 20} which made known a noninfectious, specific, serologically reactive constituent in the Brown-Pearce carcinoma²⁰ and in the V-2¹⁸ and V-7³⁰ carcinomas.

The mechanism whereby the infectious variant of papilloma virus is transformed to the masked variant in domestic rabbit tumors and rarely in cottontail tumors is not understood.⁴³ Attempts to demonstrate the presence of an inhibitor and to elucidate its nature have been made repeatedly.^{9, 10, 12, 17, 41, 47} In each instance, variable credence was given to extravasated antibody. Evidence in support^{10, 17} of this hypothesis made it apparent that antibody contributed to the masking effect. This explanation is untenable, since infectious papilloma virus is known to persist in lesions of cottontail rabbits with high antibody titer,⁴³ even in papilloma tissues which were infiltrated and covered by an extravasated serum coagulum, such as results from Roentgen irradiation in graded dosage.⁴⁷ The mechanism of alteration of the papilloma virus to a masked variant has not been elucidated. The application of a variety of experimental approaches in attempts to identify, release, or inactivate inhibitors yielded results that were not helpful. These attempts included the use of heat^{9, 10, 41} extrinsic enzymes,⁹ and the intrinsic enzymes of domestic rabbit tissues.² Shope⁴³ has pointed out the need for exploring "non-viral" tumors for the existence of biological carcinogenic agents "more elusive than the conventional tumor virus." The importance to the cancer problem of an understanding of the masking process needs no emphasis.

The Relationship of the Papilloma Virus to the Ultimate Cancer

The relationship of Shope's virus to the cancer that not uncommonly terminates the papilloma-to-carcinoma sequence is not known. Many months* after the virus initiates the papilloma, malignant transformation is observed to occur at one or more of the primary lesions. It seems unlikely that papilloma virus functions as the actuating carcinogenic agent. This conclusion resulted from a study of 33 wild cottontail rabbits with epidermoid carcinomas.⁵² These rabbits had been infected with papilloma virus many months earlier. The lesions they carried were removed at autopsy, examined histologically, and tested for the presence of infectious papilloma virus. If virus were present in lesions of these cottontail rabbits, it should be demonstrable, for it is customary to recover virus from papillomas on this host. Twelve of 40 histologically benign papillomas yielded virus but none of the 106 cancers did. Further experiments⁵³ that employed metastatic carcinomatous tissue for testing yielded convincing re-

* Papillomas induced by infection of foetal rabbits *in utero* were replaced within 7 months by epidermoid carcinomas. The papilloma-to-carcinoma sequence occurred several months sooner than it had in adult animals kept without experimental interference under similar conditions.⁵¹

EXPERIMENT III

TESTS TO DETERMINE
ACTIVE AND PASSIVE IMMUNITY OF HOST-ANIMALS
TO PAPILLOMA VIRUS

GROUP	RABBIT NUMBER	HOST-ANIMALS															CONCENTRATION OF VIRUS	CONCENTRATION OF VIRUS	DIFFERENCES IN NEUTRALIZATION TESTS BEFORE AND AFTER	(+1 OR -1 REFERS TO ONE PLACE IN DECIMAL TITRATION)
		64	65	66	67	68	69	70	71	72	73	74	75	76	77	78				
TITER OF PAPILLOMA VIRUS ON HOST-ANIMALS WHICH RECEIVED CARCINOMATOUS TISSUE																	10 ²	10 ¹		
																	10 ²	10 ¹		
NEUTRALIZATION TESTS WITH SERUM COLLECTED FROM HOST-ANIMALS BEFORE AND AFTER INOCULATION OF CARCINOMATOUS TISSUE (B = BEFORE, A = AFTER)																	10 ²	10 ¹		
																	10 ²	10 ¹		

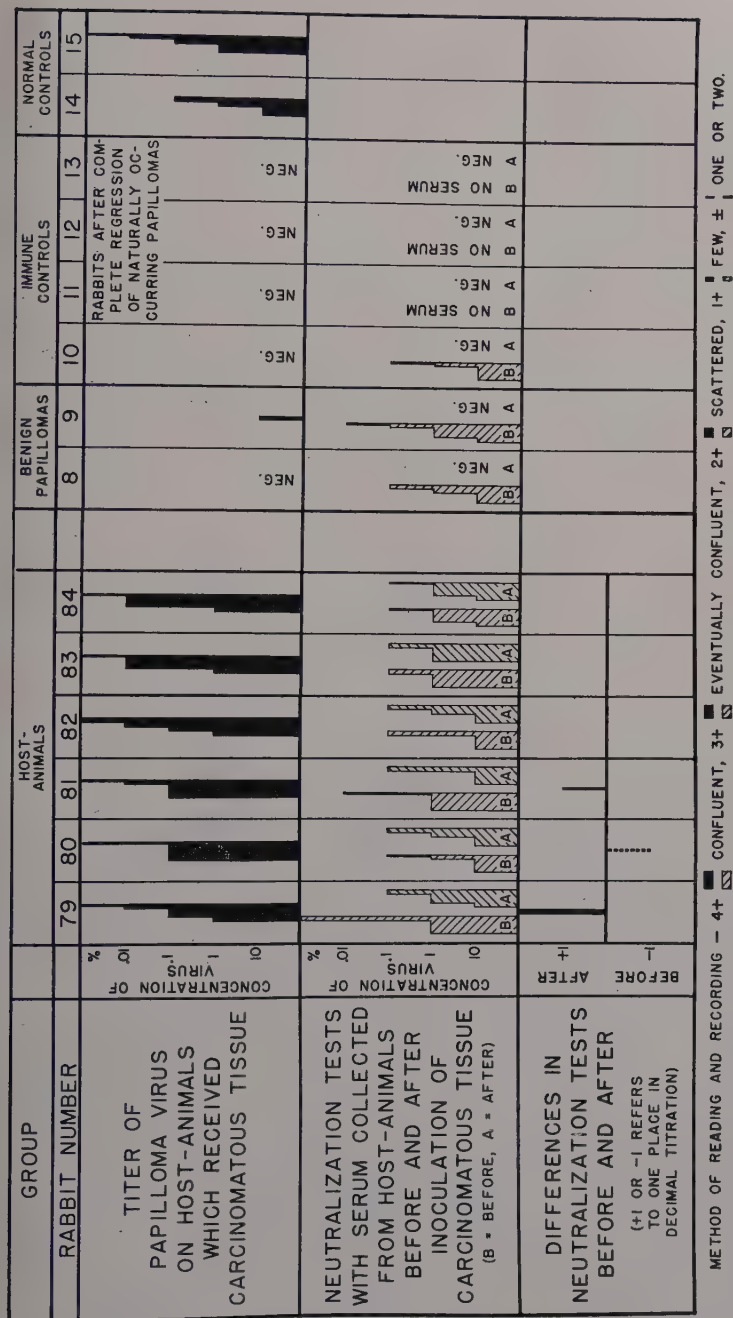


FIGURE 1. Summary of the data for the tests employed in Experiment 3 to determine whether the inoculation of cottontail carcinomatous tissue into cottontail rabbits had resulted in specific papilloma antiviral effects.

sults. Twelve metastatic cancers from lymph nodes and lungs provided the material for study. Suspensions of metastatic cancerous tissues were tested for the presence of virus by direct transfer to Eastern cottontails and to domestic rabbits and, less directly, for the ability upon multiple parenteral injection to stimulate specific antibodies for papilloma virus. 171 rabbits were employed (a) in tests for active immunity by inoculation by cross-hatch scarification of serial tenfold dilutions of virus and (b) to assess for the presence of neutralizing antibodies, acute and convalescent serum samples from each animal. If the virus were causally related to the cancer, it would be expected that metastatic carcinomatous lesions from cottontail rabbits would yield direct, or indirect, evidence for its presence. FIGURE 1 presents

SUMMARIZED RESULTS OF NEUTRALIZATION TESTS

DIFFERENCES IN INFECTIOUS TITER OF SERUM-VIRUS MIXTURES PREPARED FROM SERUM WITHDRAWN BEFORE AND 45 DAYS AFTER ATTEMPTED INDUCTION IN RABBITS OF IMMUNITY TO PAPILLOMA VIRUS BY THE INTRODUCTION OF CARCINOMATOUS TISSUE FROM COTTONTAILS.

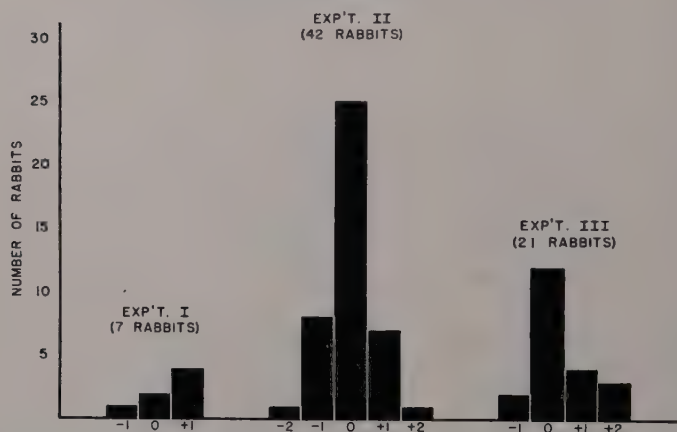


FIGURE 2. A summary for Experiments 1, 2 and 3 of the differences in infectious titer of serum-virus mixtures prepared from serum withdrawn before and 45 days after attempted induction in cottontail rabbits of immunity to papilloma virus by the introduction of carcinomatous tissue from cottontail rabbits: + = neutralization greater after than before injection of carcinomatous tissue; - = neutralization greater before than after injection of carcinomatous tissue; 0 = neutralization same before and after injection of carcinomatous tissue; 1 = 10-fold difference in final titer; 2 = 100-fold difference in final titer.

the findings of one of the four experiments that were carried out. It can be seen that evidence for the presence of virus in metastatic carcinomatous lesions was not forthcoming. FIGURE 2 presents in summary the findings of this experiment and of two similar experiments. Contrary to what we had expected when this study was undertaken, these data yielded no evidence that papilloma virus functions as an actuating carcinogenic agent. Therefore, the development of cancer was attributed to the malignant transformation of benign papilloma cells which had maintained their proliferative vigor for months.

Discussion

Are viruses actuating or provocative carcinogenic agents? Shope's rabbit papilloma virus, Bittner's mammary tumor virus, and Rous's chicken

tumor virus, as the prototypes of three large groups of tumorigenic viruses, are worthy of comparison.

The cytopathogenic properties of papilloma virus and its strict limitation to the site of tumor formation set this agent apart from the viruses of mouse mammary tumor and Rous sarcoma. Shope's virus is confined throughout the papilloma-to-carcinoma sequence to the local lesions that result where infectious virus first comes into contact with injured epidermal cells. For example, the evidence summarized in this presentation showed that the association of either variant of the papilloma virus was limited to cells at the primary site of infection and that virus was absent from the metastatic lesions that commonly result. If papilloma virus, or a detectable variant thereof, were the actuating agent of the primary squamous cell cancer, it should be found in metastatic carcinomatous tissue. On the other hand, detectable virus in an occasional cancer* or even in metastatic cancerous tissue would not be surprising. Its presence might be explained by the carry-over of a few papilloma cells in the primary cancer or by cancer cells carrying papilloma virus as a passenger. The ability of tumors to harbor, as passengers, wholly extraneous viruses for indeterminate periods of time is well known (for a review of literature, see reference 49). The extent to which this relationship can go is illustrated by the natural parasitization of the Brown-Pearce epithelioma by virus III.²⁹ Similarly, the cells of Shope's papilloma^{26, 49} and of the cancer⁴⁹ derived therefrom serve well as vehicles for a variety of wholly extraneous viruses. Seemingly incontrovertible evidence of an enduring relationship of Shope's virus to the V-2 carcinoma was reported by Kidd and Rous.^{18, 23} These workers demonstrated that transplantation of tissues for 22 passages over a period of many years resulted regularly in the development of specific antibodies in the recipient hosts. It was suggested that the virus, or a variant thereof, was in causal relationship. On test of the 46th and 50th tumor generation, however, immunologic evidence for the presence of virus was not forthcoming.⁴⁵ This finding led Kidd²¹ to conclude that papilloma virus was not essential for the continuing malignancy of the V-2 carcinoma. A second carcinoma, V-7, was reported recently³⁰ to stimulate, upon transplantation, the development of complement-fixing antibodies against the papilloma virus and to yield, rarely, papilloma virus. Since the history of the V-7 tumor resembles closely that of the V-2 tumor in early passage, an interpretation of the significance of these findings must await further studies. Hence, it seems clear from what is known concerning the relationship of Shope's virus to the ultimate cancer that there is reason for accepting, tentatively at least, rabbit papilloma virus as a provocative carcinogenic agent.

The mammary tumor virus,[†] upon transfer from mother to suckling mice, persists for months widely distributed throughout the tissues of the host.⁶ Its presence is made known in certain strains of mice by the development of adenomatous hyperplasia of mammary epithelium and mam-

* A single instance for the actual recovery of papilloma virus from a cancer was reported by Kidd and Rous,²² but "the evidence in this one case was strong that benign papilloma tissue had been present in regions which could not be subjected to such scrutiny (microscopic study) because extracted for virus test."

† For review, see "Mammary Tumors in Mice" (Moulton, F. R., editor), Amer. Assoc. for the Advancement of Science, Publication #22, 1945.

mary adenocarcinoma. The development of a spontaneous mammary tumor is attributed to the interaction of three factors: genetic susceptibility;³ hormonal stimuli; and the mammary tumor virus. The role of these three factors and of environmental influences to the development of mammary gland carcinoma of mice is not unlike the role of the determinants that operate in any other infectious disease which is dependent upon a susceptible genetic constitution.⁴⁴ The lactating mammary gland and the spontaneous mammary carcinoma provide the best sources of virus. The precise relationship of the virus to the cancer is not known. The role of hormones has been emphasized.⁴ Bonser⁵ attributes hyperplasia of mammary acini to viral activity, since she found that relief from estrogenic hormonal stimulation resulted in retrogression of the process of acinar proliferation in mice not carrying virus, whereas proliferation continued to result in intra-acinous carcinoma and, finally, infiltrating carcinoma in female mice carrying the mammary tumor virus. Hence, it appears that the mammary tumor virus differs principally from the papilloma virus in being transmitted from one generation to the next by way of milk, its wide distribution and persistence in tissues for months as an inapparent infection, and a cytotropic activity which limits evidence for its pathogenicity to the acinous epithelium of mammary glands under hormonal influence. It is not known whether the virus operates as an actuating carcinogenic agent, alters the metabolism of the host to result in a "carcinogenic hormone",⁴ or serves as a provocative carcinogenic agent in a manner similar to the papilloma virus. The failure to demonstrate the mammary tumor virus in a line of transmissible mammary cancer suggests either that spontaneous mammary cancer may occur in the absence of virus or that a masked variant of the mammary tumor virus exists. If the mammary tumor virus exists as infectious and masked variants, it should be possible by analogy with the rabbit papilloma-to-carcinoma sequence to demonstrate antibodies for the mammary tumor virus in recipients of transplants of Heston's mammary cancer line.

The agent of the Rous sarcoma satisfies the criteria of a virus and gives rise to a neoplastic growth.³¹ The virus is antigenic and apparently is widely distributed in the tissues of the host. It is held responsible for the neoplastic proliferation which makes known its presence. The genetic constitution of the host is important in determining the incidence of the tumor.¹³ The Rous agent may exist in birds for a long period of time without overt evidence for its presence.²⁷ This inapparent infection is comparable to the latent period in spontaneous mouse mammary cancer. The evidence supports the conclusion that the Rous virus is an actuating carcinogenic agent. However, the same virus that gives rise in adult chickens to neoplastic proliferation results, upon intravenous injection into newly hatched chicks, in an acute generalized hemorrhagic condition.⁷ With the development of partial resistance in the host, tumors result. These findings suggest further caution in any attempt to separate actuating from provocative carcinogenic viral agents.

Summary

The growth pattern of naturally and experimentally induced papillomas on 594 rabbits, kept without interference with the natural sequence of alterative changes, was observed to fall into three phases: proliferative, stationary, and involutionary. The termination of the natural growth pattern which resulted in the regression and disappearance of the lesions, or in malignancy and death of the host, seldom occurred until after the lesions had been under observation for six months or longer. Neoplasms developed in rabbits that were infected *in utero* as readily as in mature animals. The immediate pathogenicity of the virus and the growth pattern were not noticeably affected by the immaturity of the host or the rabbit species employed. A difference in host reactivity, however, was indicated by the early disappearance of infectious virus from domestic rabbit papillomas. The common occurrence of cancer in the natural host, the cottontail rabbit, was made known. Thirty-two of the 127 cottontail rabbits kept under observation for more than six months yielded 106 tumors which were proved to be epidermoid carcinomas by histologic study. Metastases occurred in 19 of the 32 rabbits. Attempts to recover papilloma virus from the 106 carcinomas met with failure. Nevertheless, virus was readily recovered from 12 of the 40 benign papillomas which were removed from 26 of the 32 rabbits that also had proved cancer. The relationship of Shope's papilloma virus to the carcinoma was studied by employing carcinomatous materials from Western cottontail rabbits. Eastern cottontails were utilized as the test animals, because it is the rule to recover papilloma virus from papillomas on this species of host. It was found that the transfer of carcinomatous material to normal animals did not result in an increased resistance to papilloma virus, nor did serums from these animals acquire neutralizing capacity for papilloma virus. One of the cottontails provided eight lesions for investigation: primary cancers at four sites; metastatic cancers in three lymph nodes; and a single benign papilloma. Papilloma virus was recovered from the papilloma, and a high degree of immunity resulted in the recipient test animals that received tissue, but none of the cancers from the same animals yielded evidence for the presence of papilloma virus. Hence, it was concluded that the rabbit host was without appreciable alterative effect upon the immediate pathogenicity of the virus, the tumor's growth pattern, or the development of cancer, and that papilloma virus readily infected epidermal cells to result in a neoplastic proliferative lesion, which may terminate by regression or, if sufficiently enduring, in malignant transformation to an epidermoid carcinoma. Even though papilloma virus is a remarkably efficacious carcinogenic agent for rabbits, the characteristics that make it especially worthy of investigative study are its ability to propagate and to maintain itself in proliferating epidermal cells for months, its intimate relationship to the resultant enduring proliferative lesion, and the phenomenon of masking. The properties of the rabbit papilloma virus, the mammary tumor virus and the virus of chicken tumor I, as the prototypes of three groups of viral carcinogenic agents, were discussed briefly.

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THE BERRY-DEDRICK TRANSFORMATION OF FIBROMA INTO MYXOMA IN THE RABBIT

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Infectious fibroma and myxoma of rabbits can be defined as viral infections which cause an extraordinary degree of tissue proliferation accompanied by a minimal amount of necrosis. They are not true tumors in the same sense as rabbit papilloma, for example, since the fibroma tumors run a predictable course and always regress. In myxoma also, the tumorous masses are ultimately resorbed provided the animal survives, in both cases leaving it entirely immune to subsequent reinfection.

Infectious Myxoma. Infectious myxoma was one of the earliest virus diseases to be described as such. Sanarelli²⁸ observed it in 1898, occurring in epidemic form among rabbits in Uruguay. Similar outbreaks have since been described elsewhere in Latin America,²⁴ in Southern California¹⁹ and, more recently, in Australia.²⁵ The disease is as highly contagious in nature as in the laboratory. The virus enters the body via the respiratory tract or through any wound or insect bite and is rapidly spread by the bloodstream. In the laboratory, subcutaneous inoculation of virus gives rise, within a few days, first to erythema at the site of injection, then to a soft gelatinous, hemorrhagic swelling.^{18, 27} Soon afterwards, systemic manifestations appear: low grade fever, loss of appetite, edema of the mucous membranes, difficult breathing. Death occurs 5–15 days after virus inoculation in almost every case. If the animal survives for more than a week, small tumorous masses appear in the subcutaneous tissues all over the body, particularly at the muco-cutaneous junctions. Histological examination of these tumors shows them to consist characteristically of rather large cells with abundant cytoplasm, embedded in a mucinous matrix. The nuclei are large and pale, with fragmented chromatin and often two to three nucleoli. These cells appear to arise everywhere in the body from histiocytes and from the reticulo-endothelial and vascular systems. In rapidly fatal cases of myxoma, there is little inflammatory response around these stellate cells, but in the more protracted cases, inflammation is intense.¹

The host range of myxoma is narrow and, in spite of the rapidity with which it invades all rabbit tissues, it can be shown to multiply well only in rabbits of the Genus *Oryctolagus*, and in the ectodermal and mesodermal layers of the embryonated egg.^{10, 11, 22}

Attenuated strains have been developed by Berry, by Hurst¹⁴ and by Rhodes²⁶ after repeated intracerebral passage, and by Rivers after storage in glycerol. The virus resumes its former character after passage in a more favorable tissue.

Infectious Fibroma. Closely related to myxoma is the so-called infectious fibroma of rabbits discovered by Shope,³⁰ in 1931, on the foot of a wild rabbit shot near Princeton, New Jersey. Berry repeatedly isolated a similar virus from wild rabbits in New York State some years later. In 1947, Shope was again able to re-isolate fibroma virus, the so-called Boerlage

strain. It has been isolated more recently by Kilham²⁰ from wild rabbits trapped in Maryland. The original tumors in all these isolates were composed of cells resembling fibroblasts, many of them spindle-shaped, a few polygonal, interspersed with many intercellular collagenous fibrils and inflammatory cells.¹⁶ The tumor was found to be transmissible by inoculation to domestic rabbits, in which it causes, within a few days, a well-localized tumor at the site of injection. The rabbits rarely suffer from noticeable fever or malaise, although virus can be recovered during the height of the disease from the blood, spleen and other organs, as Hurst later showed. In the domestic rabbit, the tumor is resorbed within four to five weeks, although in the wild rabbit apparently it persists much longer.

The host range of fibroma is as narrow as that of myxoma. It grows in rabbits and on the chorioallantoic membrane of the embryonated egg.³⁵

The most interesting thing about fibroma is its tendency to variation. In 1936, Shope sent a glycerinated sample of his original A isolate to Andrewes in England. Andrewes found that a change had taken place in the virus.³ It now produced small tumors after inoculation, which rapidly became infiltrated with inflammatory cells and then regressed. Further investigation by Shope³² revealed that one lot of his own glycerinated virus produced lesions, intermediate in character, between the inflammatory and original A strains. In fact, these intermediate lesions could be exactly duplicated in rabbits inoculated with a mixture of inflammatory and original A viruses. In a joint paper, Andrewes and Shope⁴ postulated these new strains to be mutants, specifically mutants with a tendency to arise repeatedly. Passage into cottontail rabbits seemed to restore to Shope's "changed" strain its former characteristics, but did not influence the inflammatory strain.

Further observation has shown that any tumor-producing sample of fibroma virus will, upon standing some months in glycerin, tend to lose not only infectivity titer but tumor-producing properties. Rapid passage will restore its infectivity but not the tumor-producing property, unless one resorts to special measures, which we shall describe later.

Relationships between Fibroma and Myxoma. The resemblances between fibroma and myxoma were sufficiently great so that further comparison was undertaken. Shope³¹ found that animals that had recovered from fibroma were solidly immune to contact infection with myxoma. In the case of animals that had recovered from fibroma infections and had been inoculated in the laboratory with myxoma, the response was not so uniform, since some animals became quite ill and a few died. It was possible, however, to pass myxoma serially in rabbits that had recovered from fibroma infection.

Careful serologic studies in several laboratories^{12, 21, 29} have shown that the antibodies induced by each of the two infections neutralize both viruses, agglutinate the elementary bodies of both, and fix complement. On the other hand, virus free filtrates prepared from myxoma infected rabbit skin or chorioallantoic membrane contain at least two soluble antigenic fractions which will induce formation of precipitating antibodies but which do not induce immunity to either virus.^{34, 37}

The Berry-Dedrick Transformation. In 1936, Berry and Dedrick,^{7, 8} stimulated by the work of Griffith and of Avery on the pneumococcus transformation, and aware of the close relationships between fibroma and myxoma, tried inoculating domestic rabbits with a mixture of fibroma virus and heat-inactivated myxoma virus. They observed that some of the rabbits inoculated with this mixture developed myxoma and were able to repeat these observations many times. Gardner and Hyde⁹, in Baltimore, after many attempts, confirmed their observations, as did also Shope,^{33, 15} Hurst and others. In all hands, however, the transformation phenomenon was troublingly irregular. Hurst,¹⁷ for instance, having successfully worked with the myxoma transformation in England, made the following comment: "When I went to Australia, one of my first attempts was to continue work on the Berry-Dedrick phenomenon. I was never able to reproduce it under Australian conditions. I have no explanation to offer."

The irregular results and the need for strict individual isolation of each test rabbit with potential myxoma have discouraged further investigation of this interesting phenomenon.

A typical transformation experiment is carried out as follows: A broth or saline suspension of fresh glycerinated or lyophilized fibroma virus is mixed in the test tube with the transforming agent derived from myxoma tissue and injected into one or more new rabbits. Within a few days, a tumor develops and is generally followed by evidence of generalized myxoma. If signs of myxoma have not appeared by the end of a week or ten days, the rabbit is killed and a portion of the tumor is ground and passed to a second rabbit. If the transformation from fibroma to myxoma is to take place at all, it is usually manifest in this second passage. If the second passage was negative a third passage is rarely positive. If myxoma does appear, it is then transmissible in series and is outwardly indistinguishable from what might be termed "natural" myxoma.

An actual experiment, resulting in complete transformation, is schematically represented in FIGURE 1. The lyophilized fibroma was of the Boerlage strain which produces good-sized tumors. It was passed once, before use in a transforming experiment. The microscopic appearance of the tissue is shown in FIGURES 2 AND 3. The tumor consists of masses of spindle cells which tend to be arranged more or less parallel to each other. The inflammatory response at this stage is slight. Under high power the nuclei are mostly oval, dark and fairly dense looking, although some are plump, with a paler nucleus, and more fragmented chromatin.

The first passage rabbit, which received the transforming mixture, developed a huge tumor consisting of masses of spindle cells in which can be seen at intervals, "nests," and strands of large stellate cells with more abundant cytoplasm. These appear to arise mainly from the capillary walls. Under high power the nuclei in these cells are also larger and pale. Nucleoli and mitotic figures are frequent. This tumor might be described as a mixture of fibroma and myxoma, the fibroma cells predominating in number (FIGURES 4 and 5).

This tumor was removed, ground, resuspended in broth and passed

directly, without further addition of transforming agent, to four rabbits. Sections of all four showed essentially the same picture, as shown in FIGURES 6 and 7. Here the cells are all far larger than those in the original fibroma tumor, their arrangement less orderly, and higher magnification shows the complete change to the picture of myxoma.

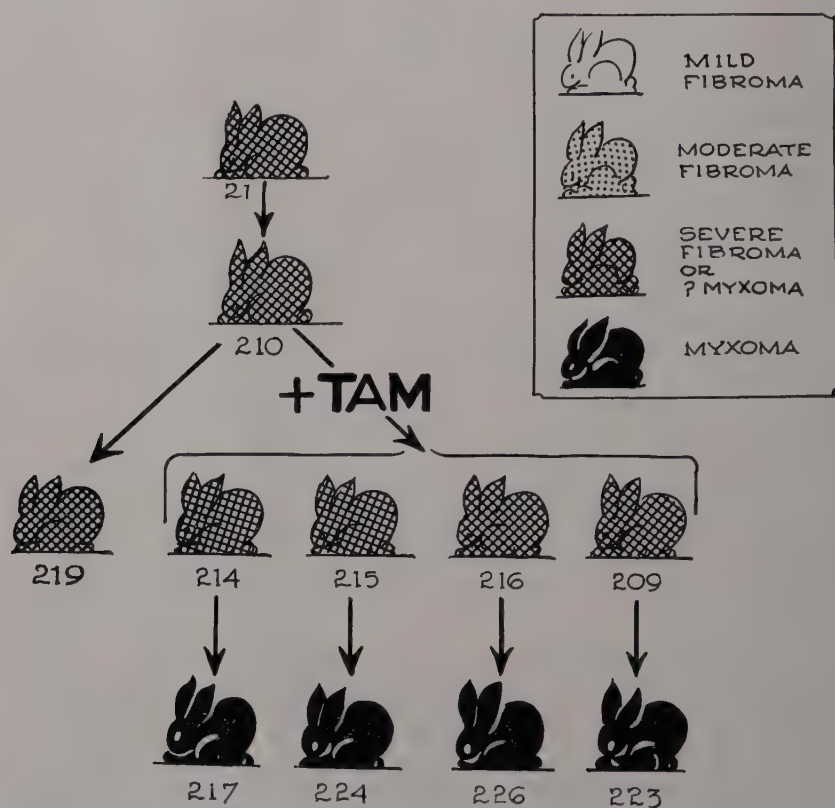


FIGURE 1. Schematic representation of a transformation experiment resulting in myxoma. TAM = transforming agent prepared from myxoma. The numbers are those of the original rabbits.

A control rabbit, which received straight Boerlage virus which had not been exposed to transforming agent, showed the same clinical and microscopic picture as the parent tumor from which it came.

Too often, however, myxoma fails to appear. In attempting to analyse these successes and failures, one can sort out the following variables: (1) the transforming agent; (2) the fibroma; (3) the new rabbit host into which the mixture is inoculated; (4) and finally, certain interrelationships such as timing, interference, immunity.

The simplest variable to control is the transforming agent. Berry⁵ showed that it can be prepared from any tissue which contains myxoma virus in sufficient quantity, whether a crude saline suspension of the tumor itself, or a water-clear suspension of elementary bodies prepared from in-

fectured rabbit skin, the chorioallantoic membrane of the developing egg. Any such suspension heated for 30-45 minutes at temperatures between

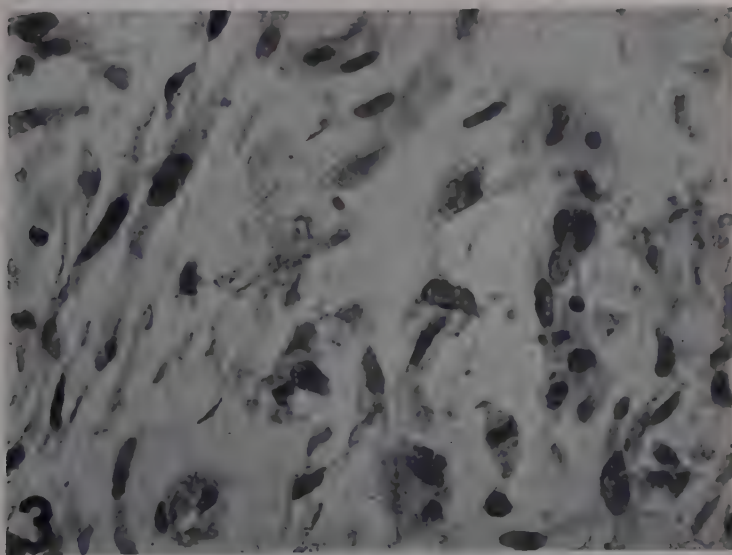
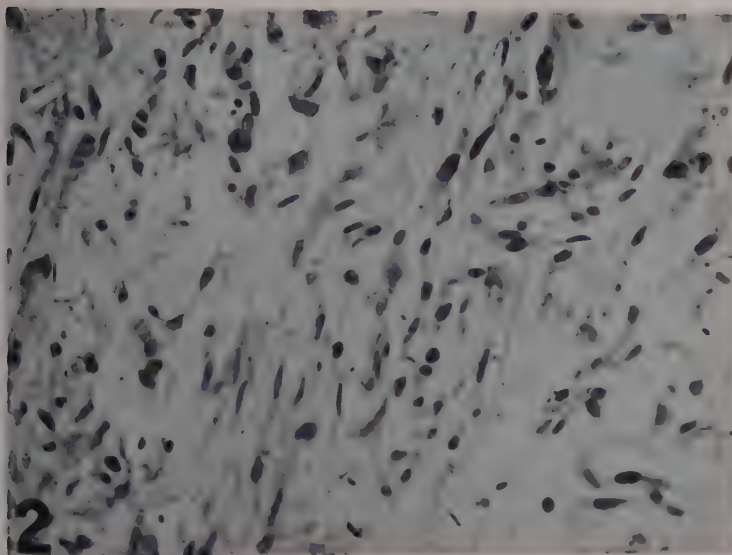


FIGURE 2. Photomicrograph of tumor tissue from Rabbit 210 (See FIGURE 1).
FIGURE 3. Higher magnification of tissue in FIGURE 2.

56°C., which kills myxoma virus, and 85°C. or so, yields active transforming principle. Our work has amply confirmed Berry's and all batches which we have prepared have been active. The material can be stored in sealed ampoules in the refrigerator for as long as a year. Moreover, the active

transforming agent can be deproteinized by repeated precipitation with chloroform. The protein free supernate, whether further treated with

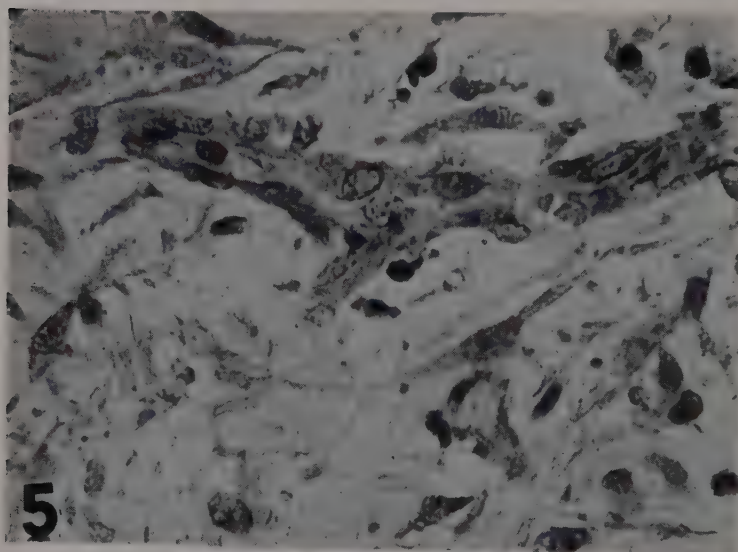
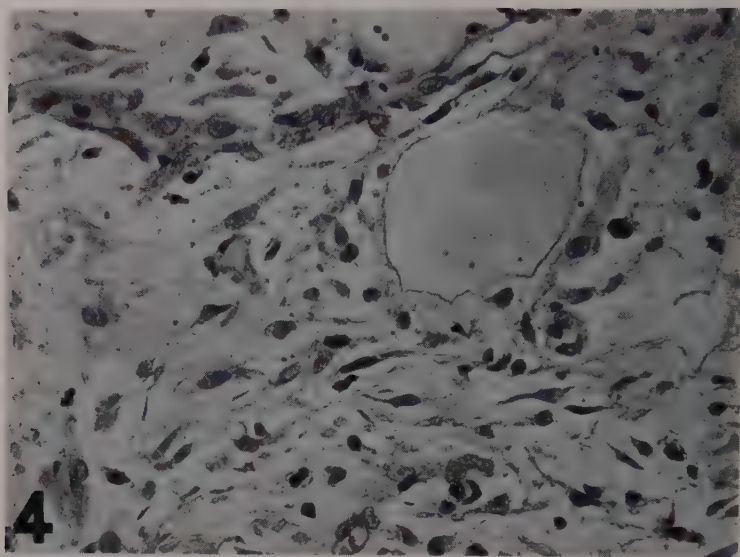


FIGURE 4. Photomicrograph of tumor tissue from Rabbit 209 (See FIGURE 1).
FIGURE 5. Higher magnification of tissue in FIGURE 4.

alcohol or not, is active in the transforming system. Analyses of this material have not yet been made, but the fact that it survives this type of chemical treatment suggests that it is a desoxyribonucleic acid.

The next factor to consider is the fibroma virus, used as starting point.

Characteristic of fresh isolates of fibroma virus is their ability to produce large vascular tumors in domestic rabbits which show under the microscope

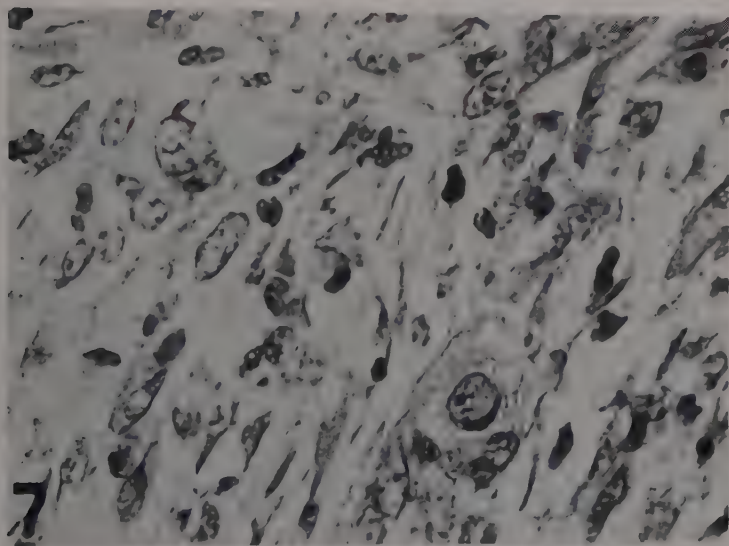
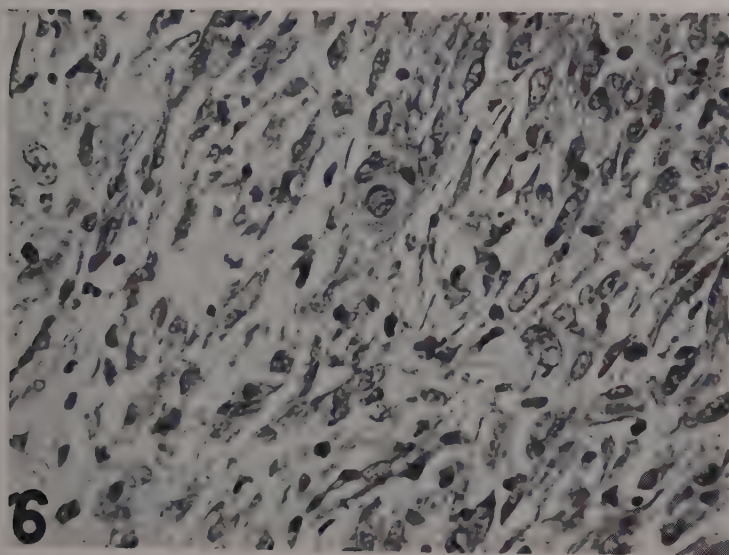


FIGURE 6. Photomicrograph of tumor tissue from Rabbit 223 (See FIGURE 1).
FIGURE 7. Higher magnification of tissue in FIGURE 6.

many spindle shaped or polygonal cells with large nuclei, as well as many slender spindle shaped cells with oval, dark nuclei. Virus maintained long in storage, however, no longer produces these large vascular tumors, but rather small tumors with scant blood supply and few or no large polygonal

cells with large nuclei. If myxoma transforming agent be added to such a strain, complete transformation to myxoma rarely ensues. The tumor, however, will be larger, hotter, more vascular and will contain more large cells with clear nuclei than did the parent strain of fibroma. Moreover, the tumor will persist weeks longer, thus resembling the wild rabbit tumor. This alteration is then transmissible from one rabbit to another, provided passage be made without prolonged storage in glycerin. So closely do these tumors resemble myxoma, that it is often impossible to recognize them with certainty without resorting to another passage. Their microscopic appearance on the other hand is characteristic enough so that one can usually tell from the number of spindle cells, stellate cells and inflammatory cells what will be the response of a rabbit inoculated with a given tumor.

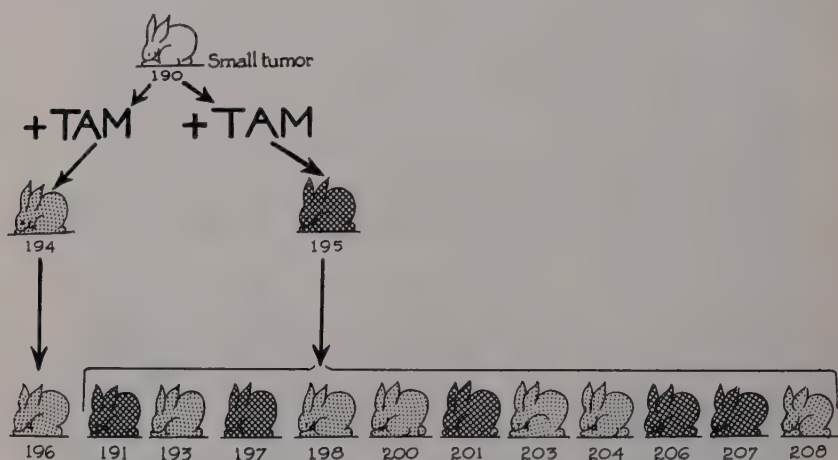


FIGURE 8. Transformation experiment resulting in "enhanced" fibroma.

An experiment resulting in enhanced fibroma only is shown schematically in FIGURE 8.

The situation here, then, would appear analogous to the stepwise transformation of pneumococcus described by MacLeod and Krauss.²³

Now, if it is possible to enhance fibroma tumor-producing properties by exposure to a transforming agent made of straight myxoma, then it should be possible to make out of an attenuated strain of myxoma, such as neuro-myxoma, a transforming agent which would enhance fibroma somewhat. Berry showed this to be feasible. We have succeeded, also, in enhancing the tumor-producing properties of the inflammatory strain of virus with a transforming agent made from Boerlage fibroma. Such a newly produced strain has never been found, however, to be any more virulent than the strain yielding the transforming agent. Berry's⁶ concept of a fibroma-myxoma "spectrum" seems the most lucid as a working basis, or perhaps better, a ladder, with the inflammatory strain of fibroma at the bottom; next, the present day strains of original A isolate which yield rather small

tumors; then, the Boerlage strain, some of the artificially "enhanced" strains, neuromyxoma; and finally, myxoma; each step capable of yielding an enhancing agent for those below it; each tending to slip down if not subjected to frequent passage in a favorable tissue.

Is exposure to virus-derived transforming agent the only means of enhancing fibroma? The studies of Ahlstrom and Andrewes² showed that rabbits treated with tar produced much larger fibroma tumors, with longer course and more numerous cytoplasmic inclusion bodies. In fact the tar-fibromas rather closely resembled wild rabbit fibromas with the enormously important difference that this alteration was not, according to Ahlstrom, heritable.

Some experiments of our own started last summer in collaboration with Dr. W. Mogabgab showed that cortisone also has powerful stimulating

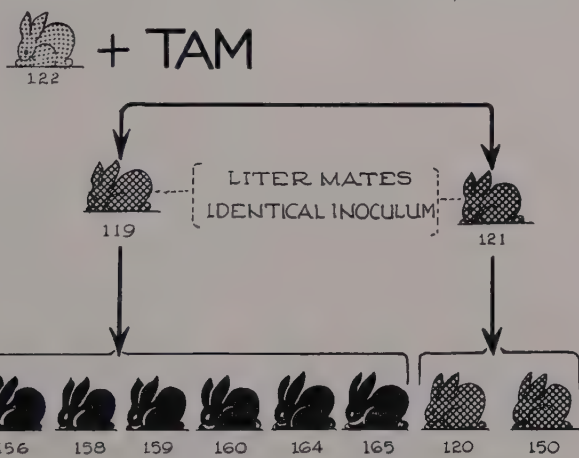


FIGURE 9. Transformation experiment with some rabbits showing severe fibroma, others myxoma.

properties for fibroma, provided the cortisone is started well before the period of maximal virus growth. The more cortisone, the larger the tumor, apparently, and the longer it will last. The tumors are not only large but extremely vascular. Whether this change is transmissible independent of further cortisone administration, and whether these tumors are more or less "transformable" than ordinary ones, we are not yet prepared to say.

Host Factors. If the degree of transformation towards myxoma depended only on the nature of the transforming agent and the tumor producing potentialities of the fibroma, then the same transforming mixture injected into several rabbits should yield the same results, but unfortunately it is not so simple and host factors are at work of which we have as yet no clear understanding. As shown in FIGURE 9, the schematic representation of an actual experiment, the identical mixture injected at the same time into littermates may produce variable results; hence the necessity for using as many animals as possible and for care in interpreting results.

Such obvious factors as age, race, sex, nutritional state of the rabbits have preoccupied us enormously at one time or another, but none of these

lines of investigation has proved consistently fruitful. We have observed complete transformation to myxoma at one time or another in newborn rabbits, in postpartum females, and in old rabbits. Our own work has been carried on almost entirely with belted Dutch rabbits, but both Berry and Hurst were quite successful with other breeds.

We thought, at one time, that temperature might be important. Careful temperature curves of our rabbits, however, have revealed no characteristic pattern of either body temperature or environmental temperature.

Finally, so far as the new host is concerned, transformations have been attempted very many times on the chorioallantoic membrane where both fibroma and myxoma viruses multiply, but none has yielded even enhanced fibroma.^{13, 36}

The problem of possible interference in some cases between the fibroma present in the inoculum and the newly forming myxoma is as yet unsolved. Hyde found that if the fibroma inoculation preceded the myxoma inoculation by as little as 48 hours, the myxoma was greatly attenuated. On the other hand, our own experiments both in eggs and in rabbits fail to show any attenuating influence of one on the other, if both are inoculated simultaneously. The myxoma always "outruns" the fibroma so to speak. The question arises, then, as to exactly when the "transformation" takes place. This question leads in turn to another: if the "transformation" takes place in some cases quite late, perhaps the newly forming myxoma is held in check by fibroma antibody. In this case, repeated passage should allow the myxoma to gain headway, but this has not proved to be the case.

Summary and conclusion. The investigations of many workers show that fibroma and myxoma are two closely related viruses, which cause great proliferation of cells of mesenchymal origin and, to a lesser extent, proliferation also of ectodermal cells. Necrosis is minimal in both diseases.

Both have a narrow host range, being limited to the rabbit and the chorioallantoic membrane.

In both diseases, recovery confers immunity not only to that entity but to the other also. Both are closely related serologically.

Fibroma, which is benign and non-contagious, can be changed into myxoma, which is lethal and highly contagious, by mixing the active fibroma with a heated extract of myxoma tumor tissue or myxoma elementary bodies.

The heated extract is active even after deproteinization.

Both fibroma and myxoma, but especially fibroma, have a strong tendency to variation. Their variants can be said to form a sort of "spectrum" or "ladder" with respect to severity.

From any variant a transforming extract can be prepared which is capable of enhancing the severity of the milder variants. This enhancement then becomes a heritable characteristic.

Certain of the intermediate fibroma variants can be enhanced by treating the host with tar and cortisone. The tar-enhancement is not inherited, the cortisone enhancement is still under study.

Any variant can be attenuated by exposure to adverse conditions such as prolonged storage in glycerin.

The same mixture of fibroma and transforming agent may behave differently in different individual hosts, so that as yet undetermined host factors influence the reaction.

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THE CROWN-GALL DISEASE*

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Crown gall is a neoplastic disease of plants that has been intensively studied in many laboratories throughout the world for almost half a century. The isolation and characterization by Smith and Townsend¹⁵ of the inciting bacterium of this disease in 1907 attracted considerable interest among pathologists generally. It should be recalled that at the time of this discovery no animal tumor had yet been produced experimentally. The now classical paper of Rous¹² on virus-induced tumors of chickens and that of Yamagiwa and Ichikawa¹⁸ dealing with the induction of tar tumors in rabbits did not appear until some years later.

In an extensive series of papers covering a period of twenty years Erwin F. Smith and collaborators¹³⁻¹⁵ stressed similarities between crown gall and malignant animal neoplasms. Crown gall was, nevertheless, not generally accepted by oncologists as being comparable to animal cancer because, as described by Smith, and more recently by others, this plant disease was a bacterial hyperplasia and, hence, not a truly independent growth. It is true that as early as 1910 C. O. Jensen,⁸ one of the leaders in experimental cancer research of that period, showed that the plant tumor tissue with which he worked was transplantable in series in the absence of any recognizable infective agent. Jensen regarded these plant tumors as being analogous to his rat sarcoma and believed that they would play an important part in future cancer research. This splendid work of Jensen's was nevertheless almost completely disregarded by other workers.

Although the presence of the specific bacterium *Agrobacterium tumefaciens* is necessary for tumor initiation, it is now known^{1, 2, 16, 17} that crown gall is not a bacterial disease in the sense that the continued development of the tumor is dependent upon the continued presence of the inciting organism. The bacterium responsible for the initiation of these plant tumors possesses the rather remarkable ability to transform normal plant cells irreversibly into tumor cells in short periods of time. Once this cellular transformation has been fully accomplished, proliferation of the altered host cells becomes an automatic process that is independent of the bacteria and their immediate metabolic products. The cells of the resulting neoplasm are characterized in many plant species by excessive powers of proliferation and very limited powers of differentiation and organization. Tumor cells of this type are transplantable and, when bacteria-free fragments are implanted into a healthy host, they develop into typical uncoordinated crown-gall tumors.^{16, 17} Since bacteria-free crown-gall tumor cells, isolated from many different plant species, have been maintained continuously in culture for periods ranging up to ten years without in any way altering their characteristic properties, it has been concluded that these are per-

* No attempt has been made to review the literature dealing with the crown-gall disease. For a general review of the problem, the reader is referred to recent articles by White, P. R., 1951, *Quart. Rev. Biol.*, 26(1):1-16; and de Ropp, R. S., 1951, *Bot. Rev.* 17(9):629.

manently altered cells that reproduce true to type and against the growth of which there is no adequate control mechanism in the host. These characters are, as far as the affected cells are concerned, the same as those by which malignant animal cells are distinguished from healthy or inflammatory cells.

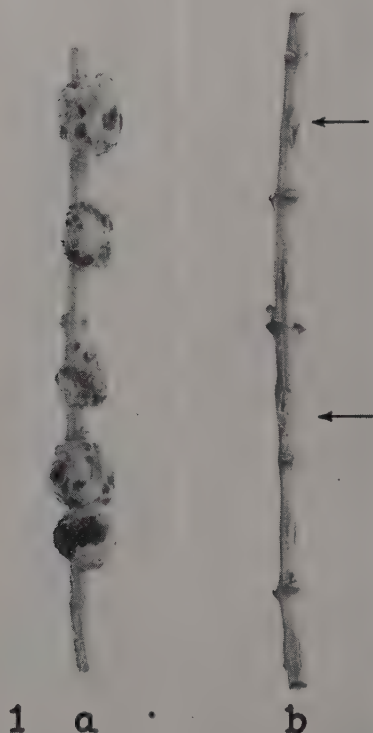


FIGURE 1. Segments of periwinkle stems inoculated with the crown-gall bacterium. The bacteria were permitted to act on the host at about 25°C. for: a, 4 days; b, 34 hours, before being destroyed by the thermal treatment. (Photographs by J. A. Carlile.)

The initiating and continuing factor or factors responsible for the alteration of normal cells to tumor cells and for the continued abnormal proliferation of the tumor cells, once the cellular alteration in crown gall has been accomplished, remain uncharacterized. The former is clearly associated with the presence of the inciting bacterium during the inception period of the disease. This factor is capable of bringing about the cellular alteration as early as 30–34 hours after the bacteria are brought into contact with susceptible plant tissues.^{1, 2, 7} When the inciting bacteria are destroyed

by thermal treatment at this early period, very small extremely slow-growing galls are produced (FIGURE 1, b). If, on the other hand, the bacteria are permitted to act on host cells for three or four days before being killed,^{2, 4} rapidly growing tumors result (FIGURE 1, a). Such tumors are not self-limiting and, when they develop on certain perennial plants, may reach very large sizes.

The alteration of normal cells to tumor cells appears, therefore, to take place gradually, leading, in a four-day period, to a rapidly growing autonomous type of cell.

Subjecting plant tissues to temperatures sufficiently high and prolonged to destroy the inciting bacteria is, of course, a drastic procedure, and another method was, therefore, developed in order to study more adequately conditions critical to the neoplastic alteration. This method² was based on the observation that normal plant cells may be converted into tumor cells by crown-gall bacteria at a temperature of 25°C. but not at 32°C. Once the cellular transformation has been consummated at the lower temperature, however, the tumor cells develop into a neoplastic growth equally well at both temperatures. It was found possible with the use of this method to bring the process of cellular alteration to an abrupt and complete halt at any desired time following inoculation by simply placing and holding inoculated plants at the relatively low temperature of 32°C. The size and rate of growth of the tumors that subsequently developed at 32°C. reflected the degree of cellular alteration that had taken place at the lower temperature up to the time that the plants were placed and held at 32°C.

With the use of this method, a number of facts relating to the process of tumor inception and development were established. The inception period was very accurately defined for two plant species.^{2, 7} In accord with results reported earlier,¹ it was found that there is a lag of 32 ± 2 hours between inoculation of the plant with the bacteria and the first evidence that the cellular alteration has taken place. Tumors of the largest size were initiated only after the bacteria had acted for 72 hours or more, while the host cells were for the most part no longer susceptible to transformation, five days or more after inoculation. This was true despite the fact that many virulent bacteria were in intimate contact with the plant cells after the fifth day. Cellular transformation in the hosts studied appeared to be dependent on a more or less unhealed wound since the alteration occurred most readily before or during the earliest stages of active wound healing.

Since the minimum period needed to accomplish the cellular alteration was found to be 30-34 hours, an attempt was made to determine whether that period could be reduced appreciably by permitting the bacteria to establish themselves in the host for 24 hours at 32°C. following inoculation. It should be recalled that the bacteria multiply as well at that temperature as they do at 25°C., yet are not capable of altering cells at the higher temperature. The results indicated that when inoculated plants were held at 32°C. for 24 hours and then placed at 25°C. for as short a period as ten hours, the cellular alteration was accomplished. Periods of less than ten hours at 25°C. were inadequate, whereas the degree of cellular alteration

became progressively greater between the ten-hour and the thirty-hour periods. The results indicate that a definite and definable threshold concentration of tumor-inducing principle is necessary to convert normal cells to tumor cells. Once this threshold is reached and passed, the reaction becomes irreversible and the tumor cells develop into a neoplastic growth.

The reason why the tumor-inducing principle associated with the crown-gall bacterium is unable to transform normal cells to tumor cells at or above 30°C. was studied. A number of possibilities do, of course, exist. The most likely appear to be either that the tumor-inducing principle is not elaborated at 32°C., or that the principle is inactivated at that temperature. This question was approached by means of an experiment in which plants were alternately subjected to temperatures of 25°C. and 32°C., following an initial incubation period of the inoculated plants for 24 hours at 32°C. to permit the bacteria to establish themselves in the host. In this experiment, five six-hour periods, or a total of 30 hours, at 25°C. were alternated respectively with one, two, four, and six-hour periods at 32°C. A continuous incubation for 30 hours at 25°C., following an initial 24-hour incubation period at 32°C., always resulted, after a suitable incubation, in large and rapidly growing tumors. The results of this experiment showed that, when six-hour periods at 25°C. were alternated with one-hour periods at 32°C., large tumors were produced in all instances. As the intervals during which the plants were held at 32°C. were increased in relation to the time of exposure at 25°C., the size and rate of development of the resulting tumors decreased. When total exposure at 32°C. approached or equaled the exposures at 25°C., occasional small tumors or, in most instances, no tumors at all developed. These results demonstrate that the tumor-inducing principle is cumulative. This is evidenced by the fact that, whereas a single six-hour exposure at 25°C. is inadequate in itself to bring about the cellular alteration, a series of such exposures, interrupted by short periods at 32°C., permits the alteration to occur. While it was not possible to determine from this experiment whether the tumor-inducing principle is elaborated by the bacteria at 32°C., the results obtained nevertheless suggest that the principle is inactivated at that temperature. If no inactivation had occurred at 32°C., the size of tumors resulting from the different exposures at 32°C. should have been much more equal in size, because a total of 30 hours at 25°C. was given in all instances. The results indicate, further, that the activity of the tumor-inducing principle was destroyed at a temperature of 32°C. at approximately the same rate that this principle was elaborated by the bacteria during a comparable period at 25°C. As a result, the residual activity of the active principle could be dealt with on a semi-quantitative basis. By shifting the balance in favor of production on the one hand and inactivation on the other, it was possible to predict within the limits of biological experimentation the eventual outcome by merely adding the number of hours that plants were held at 32°C. and subtracting this figure from the number of hours that the plants were alternately held at 25°C. It could be determined from the value obtained not only whether tumors would be initiated under a given set of experimental

conditions, but, if they were produced, the relative size of the resulting tumors could be predicted in advance with some accuracy.

Because of the excellent control that could be exercised over the transformation process, inactivation experiments were conducted over a temperature range of 23°C. to 30°C. In these experiments the temperature was controlled to within 0.02–0.03°C. The results showed that measurable inactivation of the tumor-inducing principle was limited to less than 2°C. Despite this narrow range, it was nevertheless possible to establish reaction

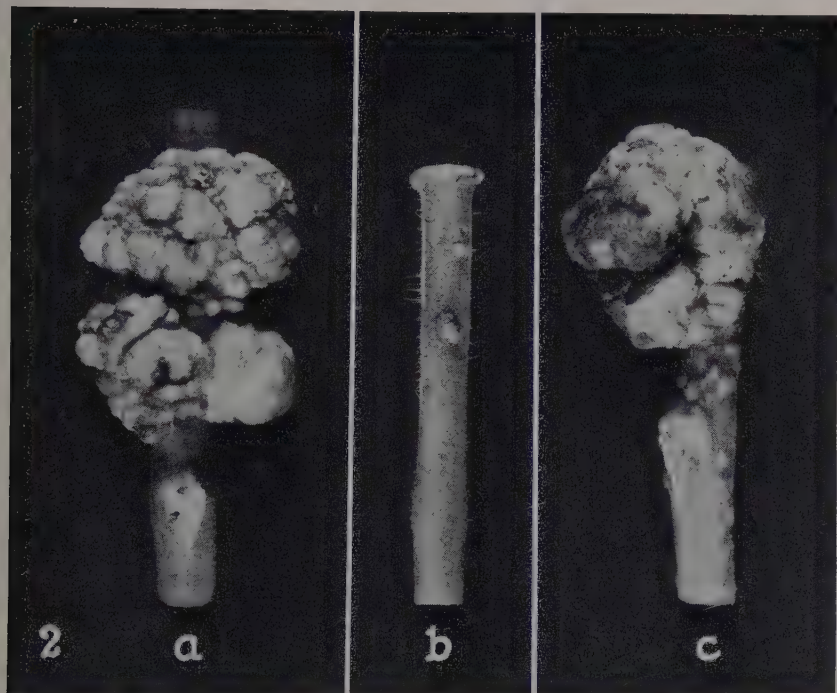


FIGURE 2. Tomato stem inoculated with: a, virulent culture; b, attenuated culture; c, attenuated culture with growth substance. A plant growth hormone was applied at the cut-stem end of the plant above the points at which the attenuated culture was introduced. (Photographs by J. A. Carlile.)

rates and, as a result, the energetics of inactivation could be studied provided one assumption is made. If it is assumed that inactivation is due to thermal destruction, then the activation energy for that destruction can be computed according to the Arrhenius equation. When the energy of activation was evaluated from the experimental results, very high values of more than 80,000 calories per mole were obtained. Since reactions of this order of magnitude are characteristic of protein denaturation, the results suggest that either the tumor-inducing principle itself or something intimately associated with its inactivation may be a factor of complex structure.

There appear to be essentially two problems of basic interest in crown

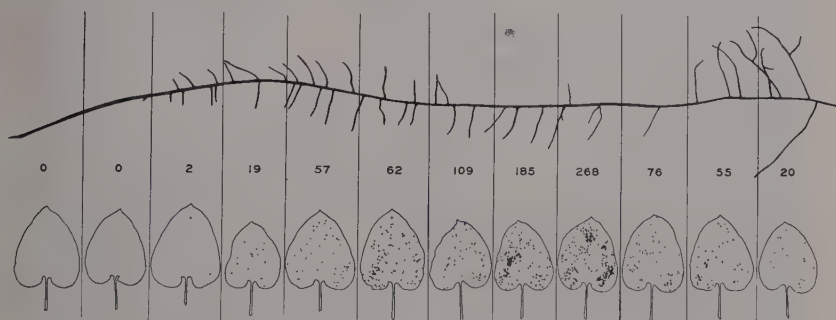
gall. The first of these is concerned with the nature of the tumor-inducing principle which, as has been indicated above, remains uncharacterized. The second problem of interest is concerned with the nature of the cellular alteration, which is a fundamental problem not only in crown gall but in other neoplastic diseases as well.

Essentially two hypotheses have been advanced to account for the continued abnormal proliferation of crown-gall tumor cells. The first of these suggests that normal cells have been permanently modified by the tumor-inducing principle into new cell types or mutants. There is a considerable body of evidence that has accumulated which indicates that a powerful growth substance is somehow concerned with tumor development. This growth-substance effect is illustrated in part by the following experiment.⁶ Isolates within strains of the crown-gall bacterium show varying degrees of virulence. Some isolates are capable of regularly initiating large tumors (FIGURE 2, a), whereas certain sister-cell cultures initiate the production of only slight cellular proliferation at the point of inoculation. Under the usual experimental conditions, the incipient overgrowths shown in FIGURE 2, b do not develop appreciably. Their growth pattern is similar to that found in tumors initiated by virulent bacteria in 34 hours. It was found, however, that when these small overgrowths were supplemented at a distance with a plant growth substance such as naphthalene acetic acid, large tumors developed that were similar to those initiated by the virulent culture (FIGURE 2, c). These tumors were transplantable. Fragments of normal tissue stimulated by the growth substance alone when implanted into a healthy host fused with the host without producing tumors. The virulence of the attenuated culture was not increased as a result of exposure to the growth substance.

Thus, it appears that at least three conditions must be satisfied for full tumor development. We need (1) the tumor-inducing principle; (2) susceptible host cells; and (3) a hormonal effect. Both virulent and attenuated cultures change normal cells to tumor cells, but perhaps to different degrees. In addition, however, a growth substance is required for full tumor development in cells transformed by the attenuated culture, while the cells changed by the virulent culture apparently generate an abundance of their own growth substance. This, together with evidence of other types, suggests that a hormonal imbalance exists in crown-gall tumor cells. It has, therefore, been postulated³ that these tumor cells have acquired, as a result of their transformation, a capacity for producing in greater than regulatory amounts a growth-promoting substance. The continued and unregulated production of such a substance could and probably would account for the continued and unregulated proliferation of the tumor cells. According to this hypothesis, the tumor-inducing principle might exert its effect by destroying in whole or in part the system that normally regulates the growth substance level within the cell or, conversely, it could act in such a way as to cause the affected cell to synthesize excessive amounts of growth substance. This interpretation, however, even if correct, gives no indication as to whether a permanent alteration of a mutational nature has resulted from the action of the tumor-inducing principle, or whether, according to the

second hypothesis of the nature of the cellular change,¹¹ the presence of some new self-duplicating factor has assumed control of the cells and is responsible for the hormonal imbalance.

There is evidence in both animal and plant literature which suggests that certain self-duplicating entities can be eliminated from cells under conditions that favor increased multiplication of those cells in relation to the multiplication of the self-duplicating factor. Preer,¹⁰ working in Sonneborn's laboratory, demonstrated that the cellular concentration of the cytoplasmic factor Kappa is determined in part by external conditions. By increasing the quantity of available food, the cells of the *Paramecium* were made to multiply faster than does the cytoplasmic particle. As a result, the concentration of Kappa per cell was reduced and the organism ultimately freed itself entirely of that particle if the culture was maintained



3

FIGURE 3. Comparison of the amounts of aucuba mosaic virus recoverable from 1 cm. segments of a 12 cm. long diseased tomato root, estimated by inoculation of each segment into a leaf of *Nicotiana glutinosa*. (From Papers Presented at 6th Ann. Meeting, Amer. Sci. Teachers Assoc., Dec. 29, 1938, Richmond, Va., p. 19, 1939.)

under conditions that favor rapid multiplication of the *Paramecium*. I have selected another example of this type from the plant literature to illustrate the point in question.* If a tomato root such as is shown in FIGURE 3 is grown in culture, it increases in length from one cm. to about twelve cms. in the course of a week. The initial one cm. fragment of the root shown above was heavily infected with aucuba mosaic virus. If, after the infected root has grown in culture for one week, it is subdivided into twelve equal parts of one cm. each and each segment is then macerated separately in water and rubbed into a leaf of *Nicotiana glutinosa*, local necrotic lesions appear in proportion to the amount of available virus in each of the twelve segments. It can be seen from FIGURE 3 that the apical two cms. of the root, in which the cells at the root tip are in a state of very active division, are free of measurable virus. The concentration of virus increases rapidly, reaching a maximum eight-to-nine cms. from the tip. It is clear, therefore, that the virus does not keep pace with the rapidly dividing cells of the root tip. With a slow-moving virus it might be possible to isolate material close to the root tip completely free of virus. Kunkel⁹ has isolated,

* The author is greatly indebted to Dr. Philip R. White for permission to use the illustration shown in FIGURE 3.

in fact, virus-free tips from rapidly growing shoots of sugar cane plants heavily infected with the mosaic disease.

This principle has been applied to crown-gall tumor cells in an attempt to determine whether they could be freed of the factor that causes them to develop abnormally.⁵ The meristematic cells of an actively growing bud, like cells found in apical regions of a root, divide with far greater frequency



FIGURE 4. Crown-gall teratoma. Note the morphologically abnormal but organized structures that develop from this type of growth. Compare with the undifferentiated and unorganized tumor shown in FIGURE 2a. (Photograph by J. A. Carlile.)

than do most crown-gall tumor cells. An actively growing shoot, which elongates as a result of the rapid division and subsequent elongation of the cells of the apical meristem, may increase in length as much as two feet in the course of a month. Rapidly growing crown-gall tumors, on the other hand, seldom reach a diameter of more than one inch in a similar period.

In order to test the hypothesis indicated above, it was necessary to obtain crown-gall tumor cells that possessed a capacity to organize buds. This was accomplished by permitting the tumor-inducing principle associated with the crown-gall bacterium to alter pluripotent cells that possessed, at the time of their alteration, highly developed regenerative powers. When such cells were inoculated there was produced at the site of inoculation, in place of the characteristic undifferentiated and unorganized crown-gall tumor, a teratoma that was composed of a chaotic assembly of tissues and organs that were highly abnormal in appearance (FIGURE 4). The

abnormal structures that developed from the complex tumor were shown to be composed of tumor cells and, hence, these structures were organized tumors. Fragments of tissue isolated aseptically from these abnormal structures and planted on a culture medium that does not support the growth of normal cells grew profusely, as did tumor cells of the type isolated and studied in the past. These tumor cells retained indefinitely a well-developed capacity to organize small abnormal leaves and buds. This organizational ability is an expression of the inherent potentialities of the pluripotent tumor cells and contrasts sharply with results obtained when cells possessing low potency at the time of alteration are transformed by the tumor-inducing principle.

Tumor shoots derived from tumor buds that developed from bacteria-free teratomata were forced into very rapid growth by a series of graftings to healthy plants. When these tumor cells were thus made to divide with great rapidity, they gradually recovered and became normal in every respect. The results obtained in this study make somatic mutation as a possible explanation of the nature of the cellular alteration in crown gall appear unlikely. They suggest, rather, that the factor that causes crown-gall tumor cells to develop abnormally becomes diluted in and is eventually lost from crown-gall tumor cells that are forced to grow and divide with extreme rapidity.

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THE STATUS OF THE SEARCH FOR A VIRUS IN HODGKIN'S DISEASE

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Hodgkin's disease is a consistently fatal tumor, which from its first recognition has been a source of controversy and speculation. Hodgkin, its classical describer, and Wilks, its early student, both found it difficult to distinguish from known infectious inflammatory processes. Reference to key review articles on this subject (Simonds,¹ Wallhauser,² Jackson and Parker³ and Hoster and Dratman⁴) show that the investigative and clinical history of this disease has been a story of repeated cycles of contrast or comparison between Hodgkin's disease and either known infectious processes or true malignancies.

At first, great energy was directed towards finding a bacterial causative organism in Hodgkin's disease. At the onset, tuberculosis was the most pursued organism and Sternberg⁵ believed that it was of etiologic importance. It was Reed⁶ who clearly concluded that this was a disease separate from tuberculosis, even though, following her observations, many investigators (Fraenkel and Much,⁷ L'Esperance⁸) still continued to probe into tuberculosis as a possible etiologic agent. It took about forty years to exhaust adequately that concept and possibility.

In those early years, other bacteria and larger organisms were encountered in patients with Hodgkin's disease and, for a time, received a variable amount of attention directed toward their etiologic significance. Much of the confusion resulted from the failure to recognize that, normally and in any disease, the lymph nodes of patients may harbor different types of organisms that do not cause a discernible disease in the person. Thus, diphtheroids received their wave of interest (Bunting and Yates⁹) and fungi such as *Torula* (Fitchett and Weidman,¹⁰ Cohen¹¹) were investigated without success. In fact, twenty years after the careful experimental work designed by the Rose Research group¹² to rule out fungi as a possible cause of Hodgkin's disease, still another report (Gendel *et al.*¹³) caused a spark in the cold prospects of relating fungi to Hodgkin's disease.

The above cycles recurred so frequently that the overall concept regarding Hodgkin's disease gradually changed. Bacteria and larger microorganisms as a cause became an empty possibility, and no further avenues of attack on the problem were apparent. Thus, the original majority of students that preferred the concept that Hodgkin's disease was an inflammatory type of specific granuloma changed to one that concluded that the disease was simply one member of the larger group of malignancies of the lymphosarcoma type. A few authors preferred to believe that, instead of being either inflammatory or neoplastic, Hodgkin's disease was a peculiar disease that lay half way between these two processes (Scala,¹⁴ Symmers,¹⁵ von Lubarsch,¹⁶ Levin¹⁷).

Nevertheless, the peculiar clinical and microscopic characteristics of

Hodgkin's disease continue to haunt the student of this disease: its cyclic behavior, the high fever and toxic picture, the proclivity for attacking younger males, the pleomorphic and often granulomatous appearance under the microscope, its associated relative energy and its unusual response to certain limited types of therapeutic agents. It is not surprising, therefore, to find that with the advent of new investigative techniques in microbiology, more especially as applied to the study of viruses, the concepts regarding Hodgkin's disease are again gradually turning towards a specific inflammatory type of process.

Hodgkin's Disease Characteristics Suggestive of or Consistent with a Virus Infection

Known virus infections often possess certain characteristic clinical, immunologic or histologic properties. When diseases of unknown cause possess some or all of such properties, they have often been shown later to have a viral cause i.e., infectious hepatitis, infectious mononucleosis, mumps, etc. Hodgkin's disease can be surveyed in order to determine how many of these characteristics it possesses.

Inclusions Bodies. Hodgkin's disease Reed-Sternberg cells characteristically possess nuclear changes of a type often seen in known virus diseases. Most striking is the large, usually eosinophilic mass of so-called nucleolar material. This structure may be phenomenally large and has many optical and tinctorial properties in common with known virus inclusions (e.g., yellow fever). Supposed "elementary bodies" will be considered later.

Relative Aergy. In many virus diseases, a state of depression of certain immunological reactions are noted. Measles and influenza are some of the most striking diseases showing this phenomenon. An increased susceptibility to infection by many bacteria is noted in these diseases. Hodgkin's disease has such characteristics, which Dubin¹⁰ studied. He reviewed the literature and noted the generally recognized high frequency of negative cutaneous tuberculin reactions (Steiner,¹⁹ Parker, Jackson, Green and Spies²⁰). He demonstrated also a relative decrease in the ability to react immunologically to stimulation by typhoid antigen, and also he encountered only one half the expected incidence of positive Wassermann tests in his cases. Forbus *et al.*²¹ encountered practically no specific antibody reaction to *Brucella antigens*, although they often cultured *Brucella* organisms from Hodgkin's disease lymph nodes. Only two possible explanations of this hypergy are apparent. Either Hodgkin's disease occurs only in persons who have such a defective immune mechanism or else Hodgkin's diseases itself causes the defect in a previously normal person.

Immunity Following Hodgkin's Disease. Many virus diseases bestow a relatively high immunity on the patient and thus re-infections are rare. In Hodgkin's disease, no information is available regarding this, since, in its neoplastic phase, Hodgkin's disease is progressively fatal. If it is an infection and if it has a non-neoplastic non-fatal phase, that phase is at present unrecognized and the status of its immunity untested.

Inflammatory Cellular Response. The leucocytic and cellular response in

most virus infections is composed of lymphocytes, plasma cells, macrophages and fixed tissue cells, rather than polymorphonuclear neutrophils. In Hodgkin's disease lymphocytes and plasma cells are prominent, as well as macrophages and fixed tissue cells. In the peripheral blood early in the disease a tendency for an increased number of monocytes occurs (Falconer²²).

Research Designed to Demonstrate Possible Viruses in Hodgkin's Disease.

Gordon *et al.*²³ was the first worker to start a systematic search for a virus in Hodgkin's disease. Before that time, others had speculated on the possible etiological role of virus in tumors in general. Their positive significance in certain tumors of animals was well known. Based mainly on clinical, inductive and some experimental evidence, several authors had considered that Hodgkin's disease might be caused by a virus (McJunkin,²⁴ Twort²⁵). Since then, many investigators have searched for a viral agent in Hodgkin's disease. Their individual contributions can best be considered under a systematic review of the different types of virus techniques used and the varieties of virus characteristics recorded.

Animal Inoculations. Repeated attempts to produce Hodgkin's disease in animals have been made. Practically no species of experimental animal has escaped inoculation. Those used include rabbits, guinea pigs, mice (Twort,²⁵ Jackson and Parker³); monkeys (Hoster,⁴ Tyzzer,²⁶ Longcope²⁷); pigeons; and chickens (Bostick and Hanna²⁸).

Every conceivable route of administration has been employed, such as subcutaneous, intramuscular, intraperitoneal, intravenous, intracerebral, splenic, lymphatics, liver, respiratory and gastro-intestinal tracts. The inoculated material has included lymph node extracts, pieces of Hodgkin's disease tissue and tissue culture. In no instance has a recognized disease been produced, nor any lesions resembling human Hodgkin's disease encountered. Some reactions have been observed, such as the lymph node hyperplasia noted by Miller and Turner²⁹ and necrotic zones in the liver encountered by Loeper and Lemaire according to Wallhauser.² Using tissue extracts and also amniotic fluid from Hodgkin's disease inoculated chicken embryos, Bostick and Hanna²⁸ failed to demonstrate any evidence of disease in chickens, guinea pigs, rabbits and rats by histologic, hematologic or clinical procedures. Guinea pigs have been noted to develop occasionally a distinct nodule upon subcutaneous injection of Hodgkin's disease ground tissues (Twort²⁵ and Gordon²³).

The sole distinct phenomenon discovered upon animal inoculation was the encephalitis response noted by Gordon²³ in rabbits. At first, the material which caused this response seemed to possess many viral characteristics: The lymph node extracts contained no bacteria; the agent was Seitz-filterable; it was inactivated by heat (80°C. for 30 min.); and the encephalitis produced had an "incubation period" of from two to six days after inoculation. The encephalitis, however, could not be passed from rabbit to rabbit. The lymph node extracts also became more potent with digestion and time. The revealing data became apparent when it was demonstrated that the encephalitic agent could be isolated from certain

normal tissues (Friedman³⁰), such as bone marrow and spleen. Further work by several authors (Turner, Jackson and Parker,³¹ McNaught³² and Edward³³) has clearly demonstrated that the factor is not a virus, that it is doubtlessly an enzyme, and that it is probably associated with the eosinophilic leucocyte.

Although the many types of animals studied have failed to show a Hodgkin's type of tissue reaction, nevertheless the extracts, sera and tissues of these animals have often been tested further for evidence of viral reactions. These characteristics will be discussed under their respective headings below.

The embryos of animals have been inoculated with Hodgkin's disease material. No Hodgkin's type of microscopic lesions have been produced. Certain phenomena, however, have been encountered. Bacteria-free extracts were noted to produce a distinctly edematous and swollen reaction in chicken embryos (Karnofsky *et al.*³⁴ and Bostick³⁵). This reaction was apparent in the first inoculations, but could not be definitely detected in later serial passes. Increased chick embryo mortality occurred when Hoster and Bechtel³⁶ placed explants of Hodgkin's disease tissue on chicken egg chorio-allantoic membranes. They noted no persistence of this effect upon subsequent egg passage, when compared to the control material. Using sterile Seitz-filtered extracts of Hodgkin's disease lymph nodes, passed serially in chicken egg amniotic sacs, Bostick³⁷ reported an increased mortality in embryonated chicken eggs. Control series inoculated with tissues other than Hodgkin's were carried along at all times. The Hodgkin's disease material was inoculated into the amniotic sac of 7-day incubated eggs and the lethal effect was recorded during the following ten days. Based on percentage alone, the mortality was only slightly greater in the Hodgkin's disease series than in the control series. Many series of eggs were used, however, and the differences in mortality were statistically very significant. No further data concerning this effect has since appeared in the literature.

Injections of ground Hodgkin's disease filtered lymph node extract and of Hodgkin's disease-inoculated amniotic fluid into both pregnant guinea pigs and guinea pig embryos has been done (Bostick³⁵). Following such inoculations, the fetuses continued to grow, were born and, at the autopsy of the newborn animals and the mothers, no abnormalities were encountered. Newborn mice 8 to 24 hours old were inoculated intracerebrally by Hodgkin's disease amniotic fluid extract and three serial intracerebral passes were made, but no clinical or microscopic evidence of disease on the part of the animals developed (Bostick and Hanna²⁸).

Tissue Culture Techniques. Two types of tissue cultures can be studied in Hodgkin's disease investigations. The older technique was the culturing of actual Hodgkin's disease tissues *in vitro* (Mankin³⁸ and Meir *et al.*³⁹). The more recent variant has been to grow normal tissue cells and then, secondarily, inoculate them with Hodgkin's disease material of various kinds.

The more classical procedure was used originally to investigate growth

characteristic of the cells in the Hodgkin's disease explant and to make special studies of the Reed-Sternberg cell. Hodgkin's disease tissue growth patterns were also compared with those of other malignancies (Lewis⁴⁰).

More recently, both procedures are being employed to study specifically possible virus element in Hodgkin's disease material. In this same sense, the cells and tissues of growing embryonated chicken eggs also are being used for growing any possible virus. The looked-for effects of a possible virus may be of two general types. One is the production of certain viral characteristics in the cells or tissue fluid (such as elementary bodies, precipitins, *etc.*). These will be considered individually in a section below. The second looked-for effect is more general and includes such phenomena as structurally "sick" or abnormal cells, unusual changes in the density or consistency of the fluid and tissues, *etc.*

Regarding these latter effects, Grand⁴¹ noted the formation of small vesicles on chicken egg chorio-allantoic membranes infected with supernatant fluid from Hodgkin's disease tissue cultures. In tissue cultures of Hodgkin's disease, an abnormal amount of cellular degeneration and liquefaction occurred. Grand⁴² prefers to interpret this as being evidence in favor of the presence of an injurious agent specific for Hodgkin's disease cultures. Rottino⁴³ also noted a liquefaction tendency that was greater in Hodgkin's disease than in other tumor tissue cultures. Although greater in and peculiar to Hodgkin's disease explants, he preferred to consider it probably non-specific and resulting from fibrinolytic enzymes liberated by the many reticular cells in Hodgkin's disease. Worken and Chambers⁴⁴ tested the effect of blood serum from Hodgkin's disease patient on tissue cultures of normal mouse lymph nodes. In 14 out of 22 cases of Hodgkin's disease the serum repressed the usual outward migration of lymphocytes and even caused lymphocytic disintegration.

After reporting on the usual characteristics of tissue culture growth of Hodgkin's disease tissues, Reiman *et al.*⁴⁵ studied in detail the effect of Hodgkin's disease, and normal sera on different types of normal, abnormal, Hodgkin's disease and neoplastic tissue cultures. Although cautious in the interpretation of their observations, they conclude that Hodgkin's disease cells and sera result in abnormal tissue cultures. Among these changes are increased fat granules in cells, more liquefactions and more numerous free cell forms. Also, more frequent nuclei in the giant cell formations and a decreased span of life of Hodgkin's disease cells were noted. These changes are essentially similar to those reported by Grand,^{41, 42} although the macromolecular particles that they encountered were considered of unestablished significance. Rottino⁴³ reports upon the frequency and prominence of giant cell formation in Hodgkin's disease, although he believes them to be of the foreign body type and not to be derived from Reed-Sternberg cells. They are quite characteristic of Hodgkin's disease tissue cultures.

Thus, three groups of investigators have noted characteristics in Hodgkin's disease exposed cells that are different from many changes noted in other types of cells. These changes can be produced by cell-free and apparently bacteria-free fluids from Hodgkin's disease tissues. These characteristics

are quite suggestive, if not distinctive, although their significance remains to be established.

Viral Characteristics of Hodgkin's Disease Tissue Material

In studying virus diseases by the well-established techniques of animal tissue and cell inoculations the detection of the presence of the virus may be most difficult. The infected cells may be generally sick and abnormal in a vague and ill-defined sense as indicated above. Often, however, certain distinct changes in the cells or their surrounding fluid can be demonstrated. Direct attention to these characteristics in regard to Hodgkin's disease can be made.

Cell Inclusions and Elementary Bodies. There is little need to distinguish between an elementary body and an inclusion structure. The former, perhaps, are more fundamental and may represent actual individual or small clumps of viruses. The latter more often represent a condensation of cellular proteins occurring in an injured cell. This condensation is usually quite characteristic of the virus under consideration. In experimental work, the presence of a known virus can be detected by the presence of such masses.

Inclusion and elementary bodies have no specific structural characteristics. They stain with a variable group of dyes and the microscopic examination presents no pathognomonic appearance. Thus, they can be easily confused with nonspecific precipitates, cell fragments and debris and artifacts. Hence, the proof of the presence of supposed elementary bodies lies, not in the finding of adequate and suggestive particulate matter in tissue material, but in establishing their viral specificity.

Although several other authors had remarked that the prominent eosinophilic masses (nucleoli?) in the Reed-Sternberg cells structurally resembled known virus inclusion masses, Gordon²³ was the first to propose that Hodgkin's disease tissues contain elementary bodies and, in his article, he shows photomicrographs of them taken by P. C. Cole. He pictures the Hodgkin's disease tissue extracts as "swarming with the bodies"⁴⁶ which are slightly larger than the elementary bodies present in vaccinia. He gives no data from which to judge the purity of the source of his supposed elementary bodies except to state that they are found in the Hodgkin's disease tissue juices.

Elementary and inclusion bodies were next described by Grand.^{41, 42} In tissue cultures, brilliant cresyl blue (11:50,000) stained inclusions, irregular in size and shape, in the Hodgkin's disease reticular, lymphocytic cells and Reed-Sternberg cells. Similarly stained cultures of normal, inflamed and lymphosarcomatous lymph nodes did not show such inclusions. In fixed tissues, Giemsa's and Seller's stains showed the inclusions in the Hodgkin's disease material and not in the controls. In cultures of normal lymph nodes, Grand noted that inclusions began to appear in the cells within 15 minutes after the addition of the supernatant fluid from a Hodgkin's disease tissue culture. By 24 hours, these inclusions were even more striking and resembled those found in Hodgkin's disease. Supernatant

fluid from other tissues than Hodgkin's disease did not cause inclusions to develop in the normal lymph node cultures.

Rottino⁴³ observed many inclusions in the cytoplasm of Hodgkin's disease tissue culture cells. He noted, however, the same in control material. He believed that phagocytosis of degenerated cell fragments would produce cell inclusions and concluded that present techniques are too crude to establish the genesis of inclusion bodies. Reiman *et al.*⁴⁵ noted cytoplasmic inclusions in Hodgkin's disease cultures, but stated that their specificity (as maintained by Grand) remains to be confirmed. Jacquez and Porter⁴⁷ made electron microscope studies of tissue culture of Hodgkin's disease and control tissue with photographic magnification up to 17,000 diameters. They did not study Reed-Sternberg cells. In the other cells however, they noted no structures suggesting virus particles. Hoster *et al.*⁴ suggest that one explanation of the inclusion bodies noted by Grand⁴¹ is that a virus of avian lymphomatosis may lie undetected in the chicken embryo substrate. Grand,⁴² however, discounts this possibility since this same chicken material was used in both control and Hodgkin's disease experiments.

Macromolecular particles from various types of lymph nodes were studied by Hoster *et al.*⁴⁸ They used a ten-step differential centrifugation procedure and then examined the particles under the electron microscope. Lymph nodes that were normal or non-neoplastic were compared with Hodgkin's disease lymph nodes and lymphosarcoma lymph nodes. They did not use the term "elementary bodies." They studied the frequency of the various sizes of particles in these tissues. In Hodgkin's disease, the predominant particle size was 10–20 $m\mu$, which differed significantly from the particle sizes found in non-neoplastic tissues. They did not comment on the significance of this finding.

Toxic Effect of Hodgkin's Disease Material. The ability of some known viruses to produce a toxic reaction when injected into certain animals is well-established. This is a different phenomenon from a simple overwhelming infection by a virus, such as was discussed above under *Animal Inoculations* (page 1164). This toxic response is supposed to be due to the formation of a specific toxin and is demonstrated very well in the case of lymphogranuloma venereum. The cultivation of that virus in embryonated chicken eggs results in yolk sac material that is toxic and lethal to mice upon intravascular inoculation. This toxin is thermo-labile, deteriorates rapidly after the death of the embryo and is most abundant in moribund eggs. A toxin has also been demonstrated in influenza-inoculated chorio-allantoic fluid.

A survey of Hodgkin's disease for the presence of such a possible toxic factor has been reported by Bostick and Hanna.²⁸ They did not use direct Hodgkin's disease lymph node extracts but studied only the amniotic fluid, a suspension of the ground chorio-allantoic membranes or yolk sac, from Hodgkin's disease inoculated chicken embryos. This fluid was inoculated intravenously into the tail veins, intraperitoneally into young mice and intravenously into the chorio-allantoic artery of 11-day incubated chicken eggs. Also intracerebral inoculations into newborn mice and young

adult mice were performed. In no instance was any suggestive evidence of a toxic factor encountered in this Hodgkin's disease material.

Precipitins and Agglutinins. These procedures might demonstrate whether an antigenic factor is associated with Hodgkin's disease. Such a factor if present would likely be of either bacterial or viral type. These procedures have been most frequently studied in connection with the many and varied types of bacteria that have been isolated from Hodgkin's disease nodes. Since, however, the etiologic relationship between bacteria and Hodgkin's disease has not been demonstrated, the data regarding them will not be reviewed here.

Twort²⁵ studied the reaction of many types of experimental animals to Hodgkin's disease lymph node tissue emulsion inoculation. Precipitin and agglutinin reactions gave indefinite results. Using human blood and urine derivatives he was unable to demonstrate agglutination and precipitin effects against Hodgkin's disease lymph node antigen material. Gordon⁴⁹ studied the reactions of the "elementary bodies" that he encountered in Hodgkin's disease material. He tested 18 specimens of Hodgkin's disease serum with several preparations of his Hodgkin's disease antigens and reported only variable results. He prepared antibodies in rabbits to his elementary bodies and obtained their flocculation.

Grand⁵⁰ inoculated rabbits with purified suspension of Hodgkin's disease lymph nodes. The nodes were prepared by differential centrifugation ($5,900 \times g$). The final purified suspension was injected intravenously into normal rabbits for 5-10 days. Later, rabbit serum was collected and inactivated at 55°C . for 30 minutes. It was tested for the presence of agglutinins for purified Hodgkin's disease lymph node extract. The readings were made under the darkfield microscope. Agglutination of very small particles was noted in the Hodgkin's disease material. Agglutination was absent in similar tests using normal, lymphosarcoma and leukemia lymph node extracts.

With amniotic fluid harvested from Hodgkin's disease-inoculated chicken eggs as an antigen, Bostick and Hanna²⁸ examined human sera for evidence of flocculation by using a modified Kline-diagnostic test for syphilis technique. No flocculation was demonstrated upon gross examination. Similar Hodgkin's disease amniotic fluid did not reveal precipitins when layered over various dilutions of normal chicken and human sera, over human Hodgkin's disease sera, or Hodgkin's disease-inoculated chicken sera. These were read by direct visual examination.

A general survey of possible serologic method was made by M. S. Hoster (quoted by Hoster and Dratman⁴). She employed the collodion particle agglutination test and the complement-fixation test in an effort to demonstrate immunologic specificity in Hodgkin's disease. Six sources of antigens which theoretically could contain a specific Hodgkin's disease antigen were used: Hodgkin's (a) tissue extracts; (b) transudates; (c) and (d) centrifugates of transudates and exudates; (e) supernatant fluid from Hodgkin's disease tissue culture; and (f) extract of embryonated chicken eggs inoculated with Hodgkin's disease tissue suspension. Several possible sources of anti-

body were examined: (a) Hodgkin's disease patients' sera; (b) Hodgkin's disease tissue extracts; (c) Hodgkin's disease injected rabbit's sera. In 10 experiments, during which 373 samples of Hodgkin's disease sera and 240 samples of control sera were examined with 96 antigen preparations, the collodion agglutination test was negative.

Complement-fixation studies reported in the literature have been few. In Hoster's experiment referred to above,⁴ she used 27 possible Hodgkin's disease antigens in testing 221 Hodgkin's disease sera against 132 control sera by complement-fixation techniques. Her results were negative.

The other efforts at complement-fixation reported in the literature have nothing to do with possible viral cause of the disease since they used as antigens various bacteria isolated from Hodgkin's disease lymph nodes (de Negri and Mieremet⁵¹ and Ayrosa *et al.*⁵² used varieties of diphtheroids).

Bostick³⁵ employed as an antigen amniotic fluid harvested from eggs in which filtered Hodgkin's disease extract had been serially passed. Complement-fixation properties were studied from two aspects. In the first instance, Hodgkin's disease patient's and normal patient's sera were tested, using the Hodgkin's disease amniotic fluid as the experimental antigen and using as a control antigen non-Hodgkin's disease serially-passed amniotic fluid. Under these conditions, 66 per cent of the Hodgkin's disease patient serum fixed more complement with the Hodgkin's disease amniotic fluid than with the control amniotic fluid, whereas only 35 per cent of the control patients' serum produced the same result. In the second instance, the Hodgkin's disease amniotic fluid was inoculated into rabbits, and their serum was later tested for complement-fixation properties by comparing the results obtained by control amniotic fluid with that of Hodgkin's disease amniotic fluid. In this study, not only was the original Hodgkin's disease amniotic fluid used as an antigen in the complement-fixation test, but also the Hodgkin's disease amniotic fluids derived from other Hodgkin's disease patients were tested. Not all rabbit sera possessed complement-fixing properties. In some rabbit sera, however, the ability to fix complement was consistently demonstrated, when using as antigens the serially passed and filtered amniotic fluid derived from multiple cases of Hodgkin's disease. These results are not spectacular, since not every Hodgkin's disease patient's serum showed complement-fixation by the amniotic fluid antigen, and the demonstration of complement-fixation properties in a rabbit following lymph node inoculations would be expected. They do reveal, however, a trend that might well be refined and amplified with profit.

Cutaneous Reactions. Intradermal sensitivity tests have been successfully employed in detecting various known virus infections. Although the Frei test in lymphogranuloma venereum is best known, significant reactions in from 65 per cent to 93 per cent of patients with known virus infections have been demonstrated in influenza (Beveridge and Burnet⁵³), vaccinia (Smith⁵⁴) and herpes simplex (Jawetz *et al.*⁵⁵).

Numerous attempts to develop a specific skin test for Hodgkin's disease have been made. Almost all of these have used as an antigen, Hodgkin's disease tissues prepared in different ways. Gordon²³ and Chapman⁵⁶ re-

ported vague, variable and erratic results that could be interpreted only as negative in their overall aspects. Bostick and Hanna²⁸ did intradermal skin injections of amniotic fluid from Hodgkin's disease injected chicken eggs. No significant or interpretable data were obtained either with the unaltered amniotic fluid or with the suspension of its sediment concentrated ten times in the ultracentrifuge at 30,000 g for one hour.

Hemagglutinins. Ever since Hirst first demonstrated the hemagglutinative abilities of influenza virus in 1941, different viruses have been systematically investigated by the use of this phenomenon. Although not specific for viruses, the technique has been a useful tool in studying viral growth, concentration and antigenic relationships.

M. S. Hoster (quoted by H. Hoster 1948⁴) found a hemagglutination factor against rabbit red cells in tissue extracts from Hodgkin's disease patients, but she also found it in control patients, and "cold" hemagglutinins encountered in Hodgkin's disease sera were no greater than in controls.

Bostick and Hanna²⁸ made a systematic study of the hemagglutinative capacity of the amniotic fluid from chick embryos inoculated with serially passed Hodgkin's disease material. The amniotic fluid, as well as ground chorioallantoic membranes plus amniotic fluid, was tested. The erythrocytes from 15 types of warm-blooded animals were tested and no hemagglutination was observed. A search was made for any possible effect that Hodgkin's disease serum might have on erythrocytes that were suspended in it before being tested with known hemagglutinative viruses. Hodgkin's disease serum did not influence subsequent hemagglutination by mumps, influenza (Lee or PR8) or vaccinia viruses. Likewise, exposure of human erythrocytes to the amniotic fluid of Hodgkin's disease inoculated eggs failed to alter in any detectable way their hemagglutination when later exposed to Newcastle, vaccinia, mumps or influenza (PR8 and Lee) virus.

Lundbach and Lofgren⁵⁷ inoculated ground lymph node emulsions into the amniotic sac of 7-day inoculated chicken eggs. Serial allantoic passages were maintained. Lymph nodes were obtained from one case each of Hodgkin's disease, Hodgkin's sarcoma, and a lymphoma. The harvested allantoic fluid showed clear hemagglutinative titres from 2.51 to 3.11 (log units). Control material did not show this. The hemagglutinative agent fixed the complement of mumps sera. Monkey inoculation resulted in a fever of 1°C. by the 16th day. They draw no etiologic conclusions regarding this agent but observe "the serological relationships between this agent . . . and mumps virus renders necessary serious consideration of the possibility of a laboratory pick-up"!

Virus Interference Phenomena. In some instances the growth of one virus is able to interfere with that of another virus. This is a limited phenomenon, for not only are there many viruses which will not affect the growth of another one on the same substrate, but even within the group of known interfering viruses strict rules must often be observed in order to demonstrate the property. Important are such factors as: which virus is inoculated first; the dose of virus; the route of inoculation; the temperature of the substrate; the number of dead virus particles; and

the condition and kinds of substrates (embryos, animals, tissue cultures, *etc.*) used, *etc.* Theoretically the simultaneous growth of viruses could result in a summation and stimulating effect on both, although this phenomena is not often recorded in the literature.

Assuming that Hodgkin's disease is an infectious viral agent, which itself is undetectable by the usual methods, it might have, however, a demonstrable effect on the growth of known viruses. In an effort to demonstrate such properties in Hodgkin's disease, Bostick⁵⁸ serially passed (at least 4 passes) Seitz-filtered extracts of Hodgkin's disease lymph nodes in the amniotic sacs of embryonated chicken eggs. The harvested Hodgkin's disease amniotic fluid was tested for virus interference properties. This was done by inoculating Hodgkin's disease amniotic fluid into 7-day incubated chicken eggs and, after three more days of incubation, a challenging dose of influenza Lee virus was inoculated into the amniotic sac. After 18 hours more of incubation, each egg was separately harvested and tested for the amount of Lee virus present by means of hemagglutination. The amniotic fluid derived from each case of Hodgkin's disease showed interference capacity on many occasions when tested, but not on every one. Sometimes, the influenza virus inhibition was complete. More often, it was simply decreased from that of carefully run control material. Of all the tests run with Hodgkin's disease amniotic fluid, 60 per cent showed ability to interfere, 27 per cent of the tests showed no interference and in 13 per cent of the tests, there was some degree of reversal (greater hemagglutinative titres in the Hodgkin's disease series than the control). The test is quite delicate and great attention to the details of technique was necessary.

Clinically, the relationships of virus infection and Hodgkin's disease have been studied or observed. The frequency of attacks of herpes infection in Hodgkin's disease has been recorded, but no influence on the disease was noted. Hoster *et al.*⁵⁹ became interested in the association between Hodgkin's disease and virus hepatitis, when two cases of Hodgkin's disease seemed to do well after such a hepatitis. They inoculated 21 Hodgkin's disease volunteers with hepatitis virus (some infectious and some serum hepatitis virus) in order to observe the effect of these agents on the progress of Hodgkin's disease. Because of the short duration of the experiment, appraisal of the results was not possible, although some favorable clinical effects were occasionally encountered. This was perhaps more true in the cases of early Hodgkin's disease. The inherent risk and experimental nature of the procedures were emphasized. Among several possible explanations for any observed amelioration of the Hodgkin's disease is the possibility that the hepatitis virus may have been successful in competing or interfering with the status of a possible Hodgkin's disease tumor virus.

Discussion and Summary

An increasingly imposing array of data is being assembled gradually which favors the concept of a virus as the cause of Hodgkin's disease. Such is the case even in the face of the fact that the disease has not yet been successfully transferred to and then identified in any experimental animal.

In man, the clinical and pathological characteristics of the disease have been long recognized as being consistent with a virus type of infection. Among such characteristics are: the state of relative anergy; the granulomatous type cellular reaction; the occurrence of morphologically acceptable inclusion bodies in the Reed-Sternberg cells; the high fever and cyclic behavior of the disease; and the tendency to attack relatively younger adults.

Much research directed towards elucidation of minute and detailed viral properties has now been done. Macromolecular, elementary and inclusion body type structures have been repeatedly described. Tissue cultures are acknowledged to show noticeable changes in liquefaction, degeneration and cell types and characteristics. Several authors have noted non-transmissible structural abnormalities in inoculated chicken embryos, although a host of other animal species have failed to show discernible abnormalities. One author reports the presence of a serially passable and filterable factor which causes a slight increased mortality in fertile chicken eggs. Toxic effects of Hodgkin's disease material on various animals have not been noted.

Studies in serologic techniques have added but little positive information. Except for one author, precipitin and agglutinin tests were negative. Complement-fixation procedures have usually been reported as negative, although one worker reported suggestive positive fixation when using amniotic fluid from eggs inoculated with Hodgkin's disease lymph node extracts. Intradermal tests have been of no value.

Many combinations and variations of tests for virus hemagglutination properties have been done. Hodgkin's disease material was found neither to possess such properties nor to be able to alter such activities in other viruses. Two sources of information suggest that a factor in Hodgkin's disease influences the activity of known viruses. One author reports that Hodgkin's disease material can interfere with the growth of influenza virus in chicken eggs and the other author detects a tendency for the hepatitis virus to influence the progress of Hodgkin's disease in patients.

It is difficult indeed to conclude that in such a series of observations, any one of which would be clear evidence of the existence of a viral type of factor, every conclusion is in error. On the contrary, it is, most likely correct to maintain that most of the observations are valid and that virus factors have been repeatedly detected in Hodgkin's disease. What research for a virus in Hodgkin's disease has not yet done is to conclusively demonstrate that these agents are present only in Hodgkin's disease tissues, that they are demonstrable in every case of Hodgkin's disease, and that they are capable of producing the disease.

Future research must be directed towards further establishing the fact that the various phenomena observed are produced by a non-bacterial agent which is filterable and which multiplies. Part of the more recent investigations have properly been careful to demonstrate such characteristics. The major obstacle, however, to obtaining absolute evidence of an etiologic virus in Hodgkin's disease is the inability so far to reproduce the disease experimentally. Until this is done, it will be impossible to

establish that a virus agent isolated even frequently from Hodgkin's disease is the cause of the tumor and is not just an unimportant secondary resident in lymph node tissues, much as the diphtheroids are in the bacterial sphere. In spite of this fundamental reservation, it is true that the pendulum of opinion is swinging again in favor of an infectious cause of Hodgkin's disease, and that an increasing mass of experimental data has demonstrated that virus type factors are intimately associated with the Hodgkin's disease granuloma.

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PRODUCTION OF NEOPLASMS BY INJECTION OF FRACTIONS OF MAMMALIAN NEOPLASMS*

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There have been many attempts to transmit mammalian tumors by injection of cell-free preparations but, at the time our studies were begun,¹ we were aware of no reported attempts to utilize isolated cytoplasmic or nuclear components of tumor cells for this purpose. Subsequently, Lettre² reported observations which suggested that isolated cytoplasmic particles, presumably mitochondria, could enter mouse ascites tumor cells, but he did not succeed in transmitting the tumor by this means.

Our studies were designed to attempt to answer the question, "Can the malignant properties and potentialities of tumor cells be transmitted to normal cells by isolated particulate components of the tumor cells?"

Materials and Methods

The following tumors were employed: (a) 2-acetaminofluorene (AAF)-induced hepatomas in Wistar rats; (b) AAF-induced hepatomas in strain AXC rats; (c) Walker carcinoma 256; (d) Murphy rat lymphosarcoma.

Preparation of Mitochondria Fraction. Five to eight grams of tissue (hepato-
toma, Walker carcinoma 256 and lymphosarcoma) were homogenized in a refrigerated Waring Blendor in a cold-room (2–4°C.), under aseptic conditions, in either 0.85 per cent NaCl solution or 0.88 M sucrose solution (dilution 1:10). The material was fractionated in a refrigerated centrifuge by the differential centrifugation technique of Claude³ as modified by Hogeboom *et al.*⁴ Cell remnants, nuclei and erythrocytes were removed by centrifuging the homogenate at 600 g for ten minutes. This procedure was repeated twice, the sediment being discarded and the supernatants pooled. The mitochondria fraction was isolated by centrifuging the pooled supernatant at 24,000 g for 20 minutes. The washed sediment was resuspended in NaCl or sucrose solution and recentrifuged at 24,000 g for 20 minutes.

Preparation of Chromatin Fraction. Under aseptic conditions, 20–30 gm. of tissue were ground with an equal weight of beach sand for five minutes, with 100 cc. of buffered (pH 7.4) 0.85 per cent NaCl solution added progressively. The triturated mixture was passed through a double layer of gauze and transferred to centrifuge tubes. The subsequent procedure was that of differential centrifugation described by Claude and Potter.⁵

Control Procedures. Smears and sections of paraffin-imbedded preparations were made from every sample of mitochondria fractions. These were stained with Janus green B, Mallory's phosphotungstic acid-hematoxylin stain and methyl green, respectively. Similar preparations of chromatin

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fractions were stained with toluidine blue, Wright's stain, methyl green, and by the Feulgen technic.

One hundred grams of normal liver were fractionated in the manner described above. The mitochondria fraction was suspended in 0.85 per cent NaCl solution containing a little methylene blue and the entire sample was examined carefully in a blood-counting chamber. Smears were made of the entire chromatin fraction of 45 gm. of lymphosarcoma tissue. These were stained with Wright's stain and examined. Samples of the packed chromatin fractions were fixed in alcohol-acetone or osmic acid, imbedded in paraffin. Serial sections were made and examined.

Experimental Procedures

AAF-Hepatomas. Hepatomas were induced in Albino Farms Wistar rats by feeding 2-acetaminofluorene. The tumor tissue was fractionated as indicated and the following procedures were employed under aseptic conditions: Saline suspensions of intact tumor cells (0.2–0.3 ml.) were injected into the liver in 23 normal adult Wistar rats at laparotomy under ether anesthesia. The surface opening was sealed with Oxycel. The mitochondria fraction was injected in the same manner in fifteen animals. Similar preparations of normal liver cells were injected in the same manner in 23 animals. Crystals of 2-acetaminofluorene, approximately 0.5 gm., were suspended in rat plasma, which was subsequently coagulated and implanted in the liver in twelve rats. All animals were sacrificed after 12–98 days.

Hepatomas were induced in AXC rats* by feeding 2-acetaminofluorene, and were fractionated as indicated. (1) Saline suspensions of intact cells were injected into the livers of previously untreated AXC rats, and pieces of tumor tissue implanted in the liver in four rats. (2) Suspensions of chromatin fraction were injected into the liver in fifteen rats.

Walker Carcinoma 256. This tumor proved to be unsatisfactory for our purpose. Intact cells and mitochondria and chromatin fractions were injected directly into mammary tissue in a few animals pretreated with estrogen or estrogen and progesterone. The abundant stroma and extensive necrosis interfered with efficient fractionation and the use of this tumor was discontinued.

Murphy Lymphosarcoma. Actively growing tumors were obtained through the kindness of Dr. J. B. Murphy and were continued by transplantation in Wistar-Carworth rats in our laboratory. Mitochondria and chromatin were prepared as indicated previously. These fractions, obtained from 20–40 gm. of tumor tissue, were suspended in 1–2 ml. of buffered 0.85 per cent NaCl solution for injection. Intact cells were injected suspended in 1 ml. of buffered 0.85 per cent NaCl solution. 1 ml. of these preparations was injected subcutaneously in the groin and, in a few instances, into the spleen, bone-marrow, thymus and liver.

* This phase of the study is being conducted in collaboration with Dr. H. P. Morris, National Cancer Institute, Bethesda, Md.

Results

AAF-Hepatomas, Wistar Rats. The pertinent data are presented in TABLE 1. Hepatomas were found at the site of injection (liver) in 2 of 118 animals injected with the mitochondria fraction. One of these had been prepared by the saline and the other by the sucrose technic. The former was found 30 days and the latter 60 days after injection.

TABLE 1
THE INCIDENCE OF HEPATOMAS FOLLOWING THE INTRAHEPATIC INJECTIONS OF
TUMOR CELLS AND ISOLATED CELL PARTICLES

<i>Material</i>	<i>Total</i>	<i>Tumor</i>
Intact cells.....	23	0
Cell residue I.....	47	0
Mitochondria (saline).....	45	1
Mitochondria (sucrose).....	73	1
Supernatant (saline).....	11	0
Supernatant (sucrose).....	27	0
Chromatin.....	15	0
Total fractions.....	218	2

TABLE 2
THE INCIDENCE OF HEPATOMAS FOLLOWING THE INTRAHEPATIC INJECTIONS OF
TUMOR CELLS AND CELL PARTICLES

<i>Material</i>	<i>Total</i>	<i>Tumor</i>
Chromatin.....	15	1 (51 days)
Cell suspension.....	4	1 (31 days)
Tissue.....	4	4 (19-31 days)

TABLE 3
THE INCIDENCE OF LYMPHOSARCOMA AND LEUKEMIA FOLLOWING THE SUBCUTANEOUS
INJECTIONS OF TUMOR CELLS AND ISOLATED CELL PARTICLES

<i>Material</i>	<i>Total</i>	<i>Tumor</i>	<i>Leukemia</i>
Intact cells.....	262	202	25 (12%)
Chromatin.....	134	44 (33%)	19 (43%)
Mitochondria.....	58	3 (5%)	1 (33%)
Chromatin and mitochondria.....	9	4 (44%)	2 (50%)

No tumors were observed after intrahepatic injection of suspensions of intact hepatoma cells, cell-residue fractions, chromatin fractions, or suspensions of 2-acetaminofluorene crystals in rat plasma.

AAF-Hepatomas, AXC Rats. The pertinent data are presented in TABLE 2.

Murphy Lymphosarcoma. The pertinent data are presented in TABLE 3. Subcutaneous tumors were usually palpable (in positive cases) in three to four days when intact cells were employed, in three to seven days with the

chromatin fraction, and in 7-18 days with the mitochondria fraction. The animals were killed after 10-30 days and the size of the tumors in those receiving intact cells and in those receiving cell fractions was found to be essentially the same. These tumors presented a remarkably uniform appearance. There was no significant difference between those induced by injection of intact cell suspensions and those which followed injection of cell fractions.¹

In the case of several of the tumors indicated in TABLE 3 as developing after subcutaneous injection of cell suspensions and of chromatin fractions, the injected material was derived from tumors that had developed after subcutaneous injection of chromatin fractions.

Discussion

The significance of these observations rests upon the establishment of two fundamental points: (a) the neoplastic nature of the induced lesions and (b) the absence of intact tumor cells in the injected mitochondria and chromatin fractions.

In the case of the AAF-induced hepatoma in Wistar rats, proving the neoplastic nature of the lesion is difficult. In an extensive experience with this tumor in the rat, we have not been able to transplant it successfully. Bielschowsky⁶ reported its successful transplantation into the Wistar rat in three instances. Establishment of the neoplastic nature of the lesions which developed in two animals at the site of intrahepatic injection of mitochondria fractions must depend upon their cytological characteristics, which were identical with those of hepatomas produced by oral administration of 2-acetaminofluorene.

Because of the possibility that failure to obtain a larger percentage of positive results might be due to impurity of this rat strain, we are continuing these studies with AXC rats. The preliminary data presented here suggest that the incidence of positive results may be higher.

There can be no doubt of the neoplastic nature of the lesions that followed subcutaneous injection of chromatin and mitochondria fractions of the Murphy rat lymphosarcoma. Their gross appearance, rate of growth, and cytological features seem to be characteristic. Definite proof is afforded by the fact that identical lesions were produced by subcutaneous injection of both cell suspensions and chromatin fractions of the chromatin-induced tumors. These "third-generation" lesions were indistinguishable from the original tumors.

We know of no way in which contamination of the mitochondria and chromatin fractions with intact cells can be excluded with absolute certainty. Direct proof of the absence of cells in these fractions is impossible. We believe, however, that the following observations indicate the improbability of such contamination:

1. Not a single recognizable cell, nucleus, or cell fragment was seen in smears or paraffin sections of the 118 mitochondria and 15 chromatin fractions of hepatoma cells, the 58 chromatin fractions of hepatoma cells,

and the 58 mitochondria and 80 chromatin fractions of lymphosarcoma cells injected into the recipient animals in this study.

2. No cells or nuclei were seen in the entire mitochondria fraction of 100 gm. of normal liver, prepared in the same manner as the hepatoma mitochondria fraction.

3. No cells or nuclei were seen in the entire chromatin fraction of 45 gm. of lymphosarcoma tissue, nor in serial sections of the packed chromatin fractions.

Suspensions were made in 1 ml. NaCl solution, of intact tumor cells, ranging in number from 500 to 6,000,000 counted in a blood-counting chamber. These were injected subcutaneously in an attempt to ascertain the approximate number of cells and the time required to produce a tumor of the size obtained with the fractionated material. It was found that a suspension of at least 3,000 intact cells in 1 ml. of NaCl solution, injected subcutaneously, was required to produce a tumor that approximated the rate of growth of the slowest growing tumor after injection of chromatin fractions.

4. When 3000 intact lymphosarcoma cells were added to the suspended chromatin fraction and the mixture was recentrifuged at 1,500 g for 10 minutes, intact cells could be readily identified in smears. It seems unlikely that such cells would have consistently escaped detection in the examination of smears and paraffin sections made routinely from every chromatin fraction.

We feel that it is highly improbable that the chromatin and mitochondria fractions were contaminated with intact cells. The evidence appears to warrant the tentative conclusion that lymphosarcoma cells were induced by this material, presumably by entrance of the chromatin strands or some component of these structures into lymphocytes of the recipient animals.

The fact that only three tumors developed in 58 animals which received injections of the mitochondria fraction of lymphosarcoma cells, in contrast to the high incidence in those receiving the chromatin fraction, raises a question as to the possibility of contamination of the mitochondria with chromatin fragments. Such contamination is indeed probable, inasmuch as the mitochondria fractions were prepared after preliminary homogenation of the tissue in a Waring Blendor, a procedure which causes fragmentation of nuclei. Chromatin strands and fragments would then be incorporated into the mitochondria fraction sedimented at 24,000 g, since they probably would not have been removed by the preceding centrifugation at 600 g. The positive results obtained with mitochondria fractions may, therefore, have been due to chromatin material. It is interesting in this connection, however, that tumors developed in 44 per cent of nine animals injected subcutaneously with a mixture of mitochondria and chromatin, as compared with 24 per cent of those receiving chromatin alone and 5 per cent of those receiving mitochondria alone. The striking difference in incidence of tumors induced by fractions of hepatoma and lymphosarcoma cells may be explicable on the basis of the difference in transplantability of these tumors in the rat.

There was a rather striking difference (TABLE 3), in the incidence of

leukemia (infiltration of liver and kidney) in animals that developed local tumors following subcutaneous injection of intact cells (12 per cent leukemia) and in those receiving cell fractions (chromatin, 43 per cent; mitochondria, 33 per cent; chromatin and mitochondria, 50 per cent). A difference of this magnitude and in this direction would not be anticipated if the development of the malignant lesions in the recipient animals were due to contamination of the cell fractions with intact cells.

If these observations are valid, they indicate that malignant potentialities of neoplastic cells reside in the chromatin material and may be transmitted to normal cells, probably by entrance of the chromatin threads or some component of these structures into the cells. The significance of mitochondria in this connection is open to question.

There is evidence that normal cells can be invaded by particles of mitochondria and chromatin. DuBuy and Woods⁷ have shown, in the case of certain variegational diseases in plants, that abnormal mitochondria appear to be capable of invading normal cells, with transmission of the disease present in the plant cells from which they were originally obtained. Horning and Petrie⁸ have also submitted evidence for intercellular migration of mitochondria.

Marshak and Walker⁹ found that suspensions of liver cell chromatin, injected intravenously, increased the rate of mitosis in regenerating liver of rats. Other fractions of the cell and a number of related substances had either no effect or an inhibitory one. Stimulation of mitosis was also produced by the fraction of chromatin soluble in 1 M NaCl. Employing P 32-labeled chromatin, they showed further that either the whole chromatin or parts of it become incorporated in the liver nuclei.¹⁰ Marshak had shown previously that the chromatin of the living nucleus is in a state of dynamic equilibrium in which portions of its nucleoprotein are constantly being removed and replaced. Since, in these experiments, the P 32 from the intravenous chromatin was incorporated rapidly into the nucleus, the results obtained suggest that this chromatin participates in the same or similar reactions. Marshak and Walker state "the possibility that extracellular nucleic acid or nucleoprotein by becoming built into the nucleus by a mechanism of physiological exchange carries with it far-reaching implications in cellular physiology and genetics. For example, replacement of part of the nucleoprotein at any one locus in the chromosome by a similar but slightly different portion from the extracellular chromatin, may lead to a change in function at the locus comparable to a gene mutation." This possibility is suggested also by the observations reported here.

Summary

Lymphosarcomas developed in rats at the site of subcutaneous and intramedullary injection of chromatin and mitochondria fractions of the Murphy rat lymphosarcoma. Leukemia occurred in a high percentage of animals which developed local tumors following subcutaneous injection of lymphosarcoma cell fractions.

Hepatomas developed in two Wistar rats at the site of intrahepatic

injection of the mitochondria fraction of AAF-induced rat hepatomas. This fraction may have been contaminated with chromatin fragments. A hepatoma appeared at the site of intrahepatic injection of the chromatin fraction in one AXC rat.

Direct proof of the absence of intact cells in these fractions is impossible, but data presented seem to indicate that such contamination is highly improbable.

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MOUSE LEUKEMIA*

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There exist inbred lines of mice in which the great majority of males and females develop leukemia after they have reached middle age. Is the development of leukemia in mice of such inbred lines caused by an obscure process of mutation, or could it be attributed to a specific pathogenic agent, transmitted from one generation to another?

Since mouse mammary carcinoma was found to be caused by a filterable agent transmitted from parents to their offspring through mothers' milk,^{1, 2} it appeared possible to assume that certain other malignant tumors may also be transmitted in a similar, though not necessarily identical, manner.³⁻⁵

The possibility was at hand, however, that in the case of certain malignant tumors, such as mouse leukemia, the pathogenic agent may be transmitted from one generation to another, not through mothers' milk, but directly through the fertilized ovum, *i.e.*, through the embryos. This assumption appeared justified because, even though leukemia occurs very commonly in successive generations of certain families of mice, no transmission of a leukemic agent through mothers' milk could be determined in these animals experimentally.⁶⁻⁸ Furthermore, leukemia is not limited to mammals, but is quite common in chickens. Because of the high familial incidence of lymphomatosis in successive generations of certain families of these birds, a direct transmission of the pathogenic agent from one generation to another through the chicken embryos had to be considered.³ The presence of the agent of chicken lymphomatosis has, in the meantime, been detected in the embryonated eggs of hens carrying the virus.^{9, 10}

Accordingly, a series of experiments has been performed on mouse leukemia with the purpose of determining whether this disease is caused by a pathogenic agent, present in leukemic cells and transmitted from one generation to another through the embryos.

In the case of mouse mammary carcinoma, the presence of the mammary carcinoma agent can be detected only by a biological assay. This consists of inoculating a biological sample, such as a tissue extract, to be tested for the presence of the tumor agent, into 10-to-21-day-old infant mice of a susceptible line, free from spontaneous mammary carcinoma, in order to determine whether these animals would develop "spontaneous" mammary carcinomas after they have reached middle age.

It was, therefore, anticipated that in mouse leukemia also, the presence of a leukemic agent could perhaps be detected by a similar biological assay, that is, by injecting material, presumably containing the leukemic agent, into newborn suckling mice of an inbred line known to have been hitherto free from this disease, with the purpose of determining whether the inoculated animals would develop "spontaneous" leukemia after they had reached middle age.

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Newborn infant mice were used for inoculations, because experimental evidence, recently accumulated, appeared to suggest that the susceptibility of mice to the inoculation with certain viruses may greatly diminish, or even completely disappear, within a few days after birth. Mice of the C3H line, or of a foster nursed C3H(f) subline, known to have had an incidence of "spontaneous" leukemia of less than 0.5 per cent for the past 18 generations, were used as test animals for the inoculations.

Mice of the Ak inbred line (FIGURE 1) that developed leukemia either spontaneously (FIGURE 2), or as a result of inoculation with Ak leukemic cell suspensions, were used as a source of the leukemic material.^{11, 12} Fragments of the considerably enlarged liver, spleen, lymph nodes, and also of leukemic tumors, of such animals, were ground with physiological sodium chloride solution to obtain cell suspensions of 20 per cent concentration.^{11, 12}

Results of Inoculations of Leukemic Cells. In preliminary experiments, an attempt was made to transfer leukemia from Ak mice into animals of the



FIGURE 1. Young, adult mice of the Ak inbred line. They remain in perfect health through early adult age, but over 70 per cent of them develop "spontaneous" leukemia at 6½ to 14 months of age.

C3H line by inoculation of leukemic cell suspensions. It appeared that, should such cell suspensions contain a transmissible leukemic agent, it would be preferable to inject leukemic cells instead of centrifugated or filtered extracts, because cell suspensions may contain such a hypothetical agent in a higher concentration.

When, however, C3H mice were inoculated with the Ak leukemic cell suspensions, it was found that newborn suckling C3H infant mice were susceptible to the implantation of such cells to such an extent that most of them developed, and died from, transplanted leukemic tumors developing within two to three weeks at the site of the inoculation.¹² This susceptibility of C3H mice to the implantation of the Ak leukemic cells appeared to be limited to the first few days of their lives, disappearing promptly after they had reached sexual maturity.^{11, 12} Adult C3H mice seemed, in most instances, to be resistant to the inoculation of large doses of the cell suspensions.

Subsequent observations of those C3H mice which had been inoculated with the leukemic cell suspensions either in early infancy, or later in life, and which did not, at first, appear to have reacted to this inoculation, revealed that frequently such mice developed generalized "spontaneous" leukemia after they had reached 12 to 27 months of age,¹³ with no evidence of



FIGURE 2. Leukemia in middle-aged Ak female. Large peripheral leukemic nodes, enormous spleen and liver, also large mesenteric leukemic tumor.

any tumor at the site of the initial inoculation. These results suggested that the leukemic cells may contain a transmissible agent responsible for the development of "spontaneous" leukemia in middle aged mice.

The leukemic cells, however, had to be considered unsuitable for the



FIGURE 3. "Spontaneous" leukemia in C3H(f) female No. 15, Exp. 1101. This mouse was inoculated subcutaneously when less than 12 hours old, with Ak leukemic centrifugated extract, and remained at first in perfect health, but developed "spontaneous" leukemia at the age of 9 months (WBC 40,750 with 75 per cent of lymphocytes). The mouse was sacrificed when moribund 2 weeks later; very large peripheral lymph nodes were found, as well as an enormous spleen, and liver, and also a large, cylindrical mesenteric tumor; the liver and kidneys were found to be infiltrated with leukemic cells. There was no evidence of any leukemic tumor at the site of the initial subcutaneous inoculation.

inoculations having the purpose of reproducing "spontaneous" leukemia, because many of the injected mice died promptly from transplanted leukemic tumors developing within a few weeks after, and at the site of, the inoculations, long before these animals might have had a chance to develop "spontaneous" leukemia.

It was anticipated that the leukemic agent, as in the case of mouse mammary carcinoma, may also be present in centrifugated extracts, and that these might be more suitable than cell suspensions for inoculation, in view of reproducing "spontaneous" leukemia in C3H mice.

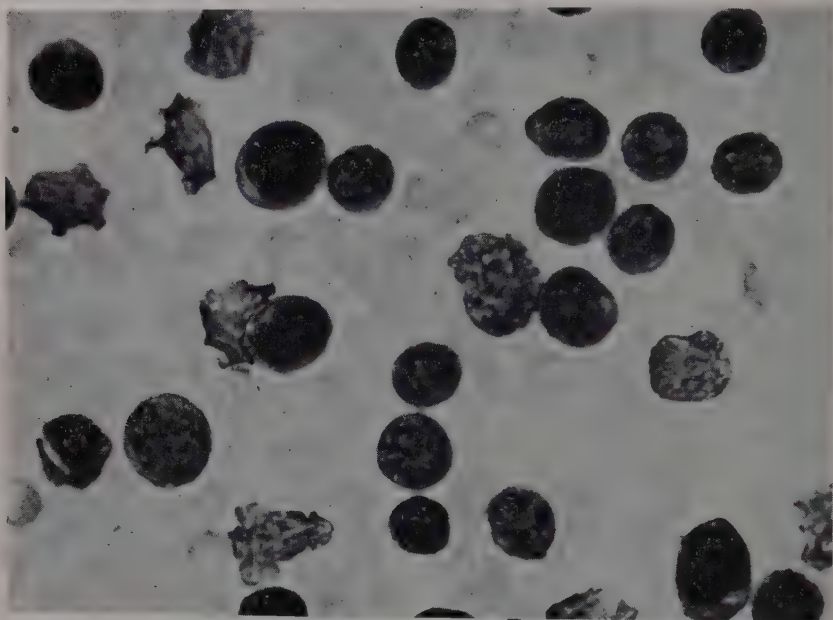


FIGURE 4. Peripheral blood smear of C3H(f) female No. 155, Exp. 1373. This mouse was inoculated when less than 1 day old with Ak leukemic centrifugated extract, and developed "spontaneous" leukemia at the age of 9½ months (WBC 496,000 with 88 per cent of lymphocytes and 12 per cent of lymphoblasts). Wright's stain $\times 900$.

Results of Inoculation of Centrifugated Leukemic Extracts. When centrifugated (3,000 RPM at 0°C. for 30 minutes)¹⁴ extracts prepared from leukemic organs of Ak mice were inoculated subcutaneously into suckling infant mice less than one day old, 29 of the injected 36 mice (81 per cent) developed "spontaneous" leukemia at an average age of 10.6 months (FIGURES 3 and 4). When 2 to 6 day-old suckling C3H mice were inoculated, leukemia developed in some of them only, at an average age of 17.8 months (TABLE 1). Up to the time of this writing, no positive results have yet been obtained in those mice which were inoculated when more than 7 days old.^{14, 15}

The susceptibility of C3H mice to the inoculation with the Ak leukemic agent, present in centrifugated extracts, appears therefore to be limited to

the first few days of life of these mice. It is also quite interesting that those inoculated within the first few hours after birth developed leukemia frequently when less than a year old, whereas those injected when a few days old, developed leukemia much later, if they developed the disease at all.¹⁵

These experiments suggest that the centrifugated leukemic extracts contain a transmissible pathogenic agent, which apparently exists in such extracts in an inactive form. When inoculated into a susceptible host, the agent remains dormant, or harmless for its host, until the host reaches middle age. At that time, for obscure reasons, the hitherto latent agent becomes activated, causing rapid multiplication of cells harboring it. This results in the development of leukemia and death of the host.

TABLE 1

"SPONTANEOUS" LEUKEMIA IN MIDDLE AGED C3H MICE FOLLOWING INOCULATION, IN INFANCY, WITH CENTRIFUGATED AK LEUKEMIC EXTRACTS

<i>Age in days when inoculated</i>	<i>Number of mice inoculated</i>	<i>Number of mice developing leukemia</i>	<i>Per cent developing leukemia</i>	<i>Average age in months when leukemia appeared</i>
1	36	29	81	10.6
2 to 6	22	12	55	17.8

TABLE 2

"SPONTANEOUS" LEUKEMIA IN MIDDLE AGED C3H MICE FOLLOWING INOCULATION, IN INFANCY, WITH FILTERED AK LEUKEMIC EXTRACTS

<i>Age in days when inoculated</i>	<i>Number of mice inoculated</i>	<i>Number of mice developing leukemia</i>	<i>Per cent developing leukemia</i>	<i>Average age in months when leukemia appeared</i>
0.5	25	8	32	8.5
8	7	2	29	25

Results of Inoculation of Filtered Leukemic Extracts. That the leukemic agent is filterable was demonstrated in a series of experiments, in which cell suspensions prepared from Ak leukemic organs were centrifugated and then filtered through Seitz (S-1) filters. The resulting filtered extracts were then inoculated into 32 suckling infant mice of the C3H line. As a result, 10 of them developed leukemia (TABLE 2).

Presence of the Leukemic Agent in Normal, Healthy Ak Embryos. Since mouse leukemia develops in successive generations of mice of the Ak line, and since it is not transmitted through the milk,^{6-8, 13*} it appeared reasonable

* That leukemia may develop also in those Ak mice which did not ingest a single drop of their mothers milk, was evident from the following experiment:¹³ A litter was removed by Caesarean section from a healthy pregnant (at term) Ak female mouse, and was transferred, for nursing, to a foster mother of the C57 (black) line, known to have been free from spontaneous leukemia for at least 15 generations. Of these transferred and foster nursed, Ak infant mice, 3 survived; 2 of them developed later on "spontaneous" leukemia at 6 and 10 months respectively, and 1 is still at good health at 11 months of age. These three foster mice had 13 offspring; eleven of them developed leukemia at an average age of 8.1 months. The results of our experiment were therefore essentially consistent with those reported previously by MacDowell, Richter,⁶ Barnes,⁷ Furth,⁸ and their associates.



† FIGURE 5. "Spontaneous" leukemia in C3H(f) female No. 118, Exp. 1155. Very large peripheral lymph nodes, enormous spleen and liver, very large cylindrical, mesenteric tumor, also large mediastinal tumor. This mouse was inoculated, when less than 12 hours old, with a cell suspension prepared from normal, healthy Ak embryos. The mouse remained, at first, in perfect health, but developed leukemia at 8½ months of age (WBC 260,000 with 65 per cent of lymphocytes).¹⁴

to assume that the transmission of the leukemic agent, if it occurs at all, may take place directly through the embryo. In order to determine whether the Ak embryos actually contain the leukemic agent, a series of experiments was carried out in which normal, healthy Ak embryos were removed aseptically from the uteri of normal, young, pregnant Ak females. The animals, used as donors of the embryos, were in perfect health at the time they were operated on. Their ancestors, however, had died from spontaneous leukemia.

These normal embryos were ground with physiological sodium chloride solution, and the resulting cell suspensions were then inoculated into newborn suckling infant mice of the C3H line.¹⁴ Thirteen C3H mice were inoculated when less than one day old, and seven of them developed "spontaneous" leukemia at an average age of 11.9 months (FIGURE 5). Forty-three C3H mice were inoculated when two to seven days old, and 11 of them developed leukemia at an average age of 17.6 months.¹³ A phenomenon similar to that observed in experiments dealing with the centrifugated leukemic extracts

TABLE 3
"SPONTANEOUS" LEUKEMIA IN MIDDLE AGED C3H MICE FOLLOWING INOCULATION,
IN INFANCY, WITH AK-EMBRYO-CELLS

<i>Age in days when inoculated</i>	<i>No. of mice inoculated</i>	<i>No. of mice developing leukemia</i>	<i>Per cent developing leukemia</i>	<i>Average age in months when leuk. appeared</i>
1	13	7	54	11.9
2 to 7	43	11	26	17.6
9	6	0		

was observed in this series; namely, that the susceptibility of the C3H(f) infant mice to the inoculation of the leukemic agent was actually limited to the first few days of their lives and that the earlier the infant mice were inoculated, the earlier they developed spontaneous leukemia. Among those inoculated with the Ak embryo cell suspension, a number of those injected when less than 12 hours old developed spontaneous leukemia at 8½ months of age,¹⁴ whereas those injected when they were more than 2 or 3 days old developed leukemia also, but not before they reached 12 to 20 months of age¹³ (TABLE 3).

Transmission of the Leukemic Agent from Parents to Offspring. Once inoculated into newborn, suckling mice of a hitherto leukemia-free, but apparently susceptible line, the leukemic agent not only infected most of the inoculated animals, causing in them the development of "spontaneous" leukemia, but also passed from the inoculated carrier-hosts into their offspring. Some of them, in turn, later on, also developed leukemia, and died from it. This was evident from the following experiments:¹⁴

Suckling infant C3H, or C3H(f), mice were inoculated with either centrifugated leukemic extracts, with leukemic cell suspensions, or with normal Ak embryo cell suspensions. After the inoculated infant mice had reached

sexual maturity, they were mated, and were designated as "inoculated parents"; the resulting offspring were not treated in any way, but were kept under observation. While these experiments are still in progress, the following results have been obtained up to the time of this writing: Of the 18 inoculated parents, 12 developed leukemia at an average age of 13.6 months. Of the 46 untreated offspring, 17 (*i.e.*, 37 per cent) have developed leukemia at an average age of 16.5 months (TABLE 4).

Since the leukemic agent apparently passes from the inoculated parents to their offspring, causing the development of leukemia in both these generations of mice, it is possible to assume that a similar phenomenon may take place, later on, in subsequent generations of mice. If this assumption is correct, it would then logically follow that the leukemic agent would continue the "vertical"* trend of transmission,^{3, 4} passing through the embryos from one generation to another, in this particular line of mice. In other words, by inoculating infant mice of the C3H, or C3H(f) line, with

TABLE 4
"SPONTANEOUS" LEUKEMIA IN MIDDLE AGED C3H MICE FOLLOWING INOCULATION OF THEIR PARENTS WITH AK LEUKEMIC EXTRACTS

	No. of mice observed	No. of mice developing leukemia	Per cent developing leukemia	Average age in months when leuk. appeared
Inoculated parents.....	18	12	67	13.6
Untreated offspring.....	46	17	37	16.5

the leukemic agent, either by injecting the leukemic cells, centrifugated leukemic extracts, or normal Ak embryo cell suspensions, we may be able to change a line of mice hitherto essentially free from this disease into a family of mice in which leukemia may from now on develop "spontaneously" in successive generations, as in the case of the leukemic Ak line. The possibility, however, must be considered that the leukemic agent may become adapted to the new strain of mice to such an extent that it may, after having passed through one or two generations, fail to become activated even though it may be transmitted through the embryos from one generation to another, and even though it may be carried by the new hosts.

It must also be considered that various strains of mice may differ in susceptibility to a given leukemic agent. Mice of the C3H line are apparently susceptible to inoculation, under certain experimental conditions, with the Ak leukemic agent. Mice of other strains, however, may be less susceptible to the inoculation with the same leukemic agent under apparently identical experimental conditions.¹³

Various Properties of the Mouse Leukemia Agent. Various biological prop-

* The term "vertical" transmission was suggested^{3, 4} to designate the passage of pathogenic agents from one generation to another (rickettsia through eggs in ticks, mosaic virus through seeds in plants, mammary carcinoma virus through milk in mice). On the other hand, "horizontal" transmission refers to the passage of pathogenic agents from one host to another within the same generation, through contact, food, or intermediary carriers (foot-and-mouth disease, typhoid fever, etc.).

erties of the mouse leukemia agent are now being investigated, such as its ability to survive, *in vitro*, in the refrigerator, at room temperature, and at either 42° or 56°; its pathogenic properties when inoculated in very small doses; its antigenic properties; its possible presence in various organs of the leukemic carrier mice, *etc.* These experiments are still in progress, and will be reported later.¹³ It is already evident from these studies, however, that the agent can be preserved, *in vitro*, in centrifugated Ak leukemic

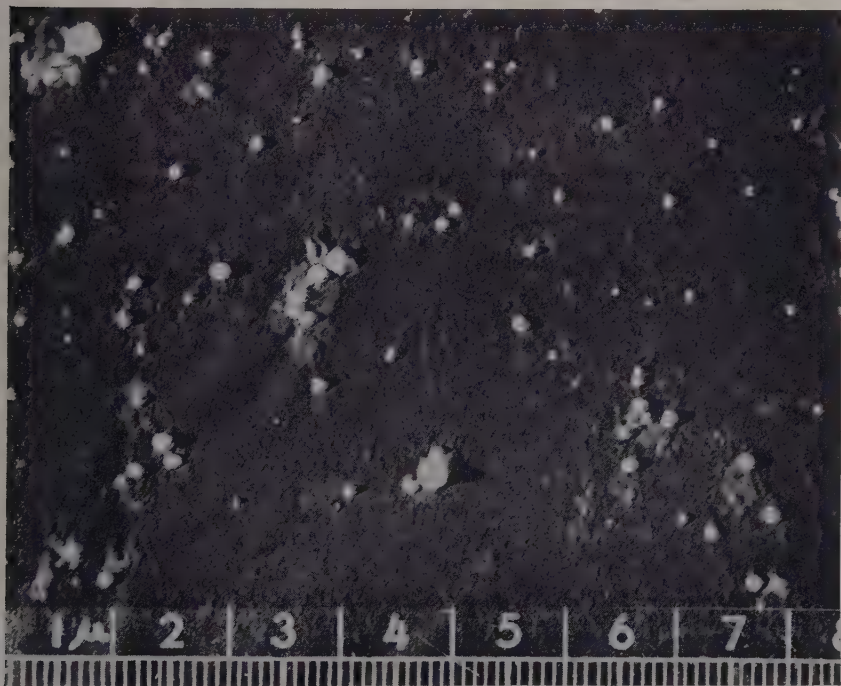


FIGURE 6. Electron-micrograph of mouse leukemia. Cell suspension of 20 per cent concentration prepared from liver, spleen, and peripheral nodes of a leukemic Ak mouse in phosphate buffer pH 7.4 (0.2 M ionic strength) was centrifugated at 1,400 g for 30 minutes; the supernatant was then removed, and centrifugated at 144,700 g for 25 minutes. The resulting pellet was resuspended in the same phosphate buffer, and segregated by electrophoresis (anodic). Chromium shadowed. $\times 15,000$. Numerous spherical particles varying in size from 20 to 200 $\mu\mu$.

extracts, at room temperature (21°C.) for 7 hours and, in the refrigerator, at 0°C., for 72 hours.

Experiments on the dosage of the Ak leukemic agent suggest that dilutions up to 1:10,000 of the leukemic cell suspensions of the original 20 per cent concentration, were still pathogenic for newborn infant mice of the C3H line; thus 2 of 4 C3H mice, inoculated, when less than 1 day old, with the 1:10,000 dilution, developed "spontaneous" leukemia at 18 and 19 months respectively.

Electron Microscopic Examination of Mouse Leukemia. A series of experiments was carried out with K. S. McCarty and I. J. Cohen, in which,

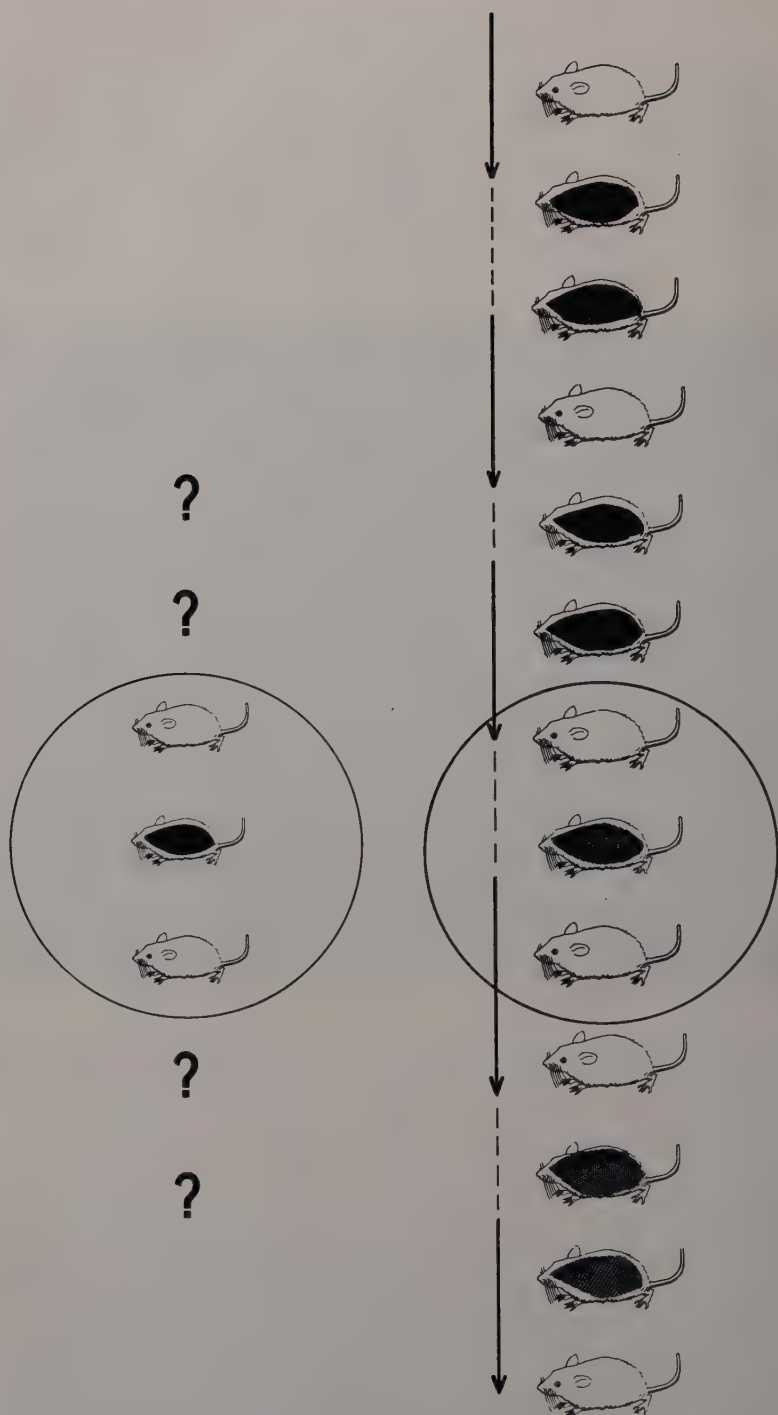


FIGURE 7. Mouse leukemia may appear to be a "spontaneous" disease, if the information available is limited to the leukemic mouse (black shadowed), and to one preceding, and one succeeding, generation.

FIGURE 8. The "vertical" transmission of mouse leukemia becomes discernible only when records are available covering a sufficient number of successive generations. Not all mice, carrying the leukemic agent, develop symptoms of disease; some of them may die without signs of leukemia, before they reach the leukemic age, even though they carried, and transmitted, the agent.

mouse leukemia extracts, segregated by differential ultracentrifugation followed by electro-phoresis, were examined with the electron microscope by K. S. McCarty.¹⁶ In all samples examined, large quantities were found of small spherical particles having a high density to the electron beam and a diameter varying in size from 20 to 200 m μ (FIGURE 6). In several experiments the filtered extracts containing the particles were injected into newborn infant mice of the C3H(f) line and, at least some of the inoculated animals developed leukemia after they have reached 6½ to 12 months of age.^{13, 15, 16} It is, therefore, quite possible that these particles actually represent the mouse leukemia agent. Additional experimental evidence, however, is still needed to justify such an assumption.

Conclusions. Experiments thus far performed suggest that leukemia which develops "spontaneously" (FIGURES 7 and 8) in mice of the Ak inbred

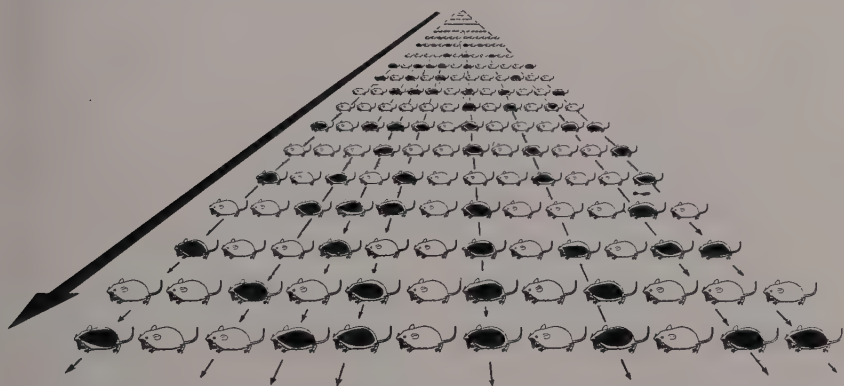


FIGURE 9. Vertical transmission of mouse leukemia. Black shadowing indicates those mice which developed leukemia. Each horizontal row indicates one generation. The leukemic agent is communicable from one individual to another of successive generations, through the embryos. In most instances the leukemic agent remains in its inactive form, causing no symptoms of the disease. In some instances, the leukemic agent becomes activated in middle-aged carriers, causing symptoms of leukemia.

line is caused by a filterable agent. This agent is transmitted from one generation to another (FIG. 9) directly through the embryos. Carrier mice, earmarked to develop leukemia later on, are in perfect health through early adult age. The leukemic agent apparently exists in such carriers in a dormant form, harmless for the hosts. After the carrier-hosts have reached middle age, however, the agent becomes activated. The activation of the leukemic agent is prompted by some, as yet unexplained, reasons. The activated agent causes rapid multiplication of cells harboring it, resulting in leukemia and death of the carrier-host.

The leukemic agent is present in cell suspensions, as well as in centrifugated or filtered extracts, prepared from liver, spleen, lymph nodes and leukemic tumors removed from Ak mice that developed leukemia. Cell suspensions prepared from normal, healthy Ak embryos, also contain the leukemic agent.

The agent can prompt the development of "spontaneous" leukemia in middle aged mice of a line (C3H) hitherto essentially free from this disease,

provided that it is inoculated in early infancy. The susceptibility of C3H mice to the inoculation with the Ak leukemic agent disappears, in most instances, within a few days after birth. When inoculated into suckling C3H infant mice, the agent not only causes the development of leukemia in most of the inoculated animals, but is transmitted to their untreated offspring, causing also, at least in some of them, the development of "spontaneous" leukemia, after they have reached middle age.

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THE TRANSMISSIBLE VENEREAL TUMOR OF DOGS: OBSERVATIONS ON FORTY GENERATIONS OF EXPERIMENTAL TRANSFERS

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The transmissible tumor of dogs is a neoplasm which occurs usually on the external genitalia of either sex and which is transmitted naturally by coitus. It is transmissible experimentally using entire, viable cells only. The tumor is usually referred to as a sarcoma or as a lymphosarcoma, prefaced by such terms as "venereal," "infectious," "contagious," "transmissible," or "transplantable." It has also been called contagious or venereal granuloma and even canine condyloma. Mulligan^{1, 2} has introduced the term "histiocytoma" to include the transmissible venereal tumor of dogs. In some of the European literature this neoplasm is called "Sticker's sarcoma" in reference to the extensive early work published by this author.³⁻⁶

Data concerning the distribution and incidence of transmissible venereal tumor in dogs are meager, but there is some suggestion in the literature that there may be geographical differences. The tumor has been reported in many European countries, North America, South America,⁷ Japan,⁸ China,⁹ and Java.¹⁰ According to Seligmann,¹¹ it was present in the native dogs of New Guinea before the arrival of Europeans. Seligmann also saw a case in Ceylon. It is reported¹² that the transmissible venereal tumor of dogs is being seen with increasing frequency in the southwest of France, where it occurs in some valleys in epizootic proportions. In some villages within the area every dog is said to be affected.¹³ In the veterinary surgical clinic at Messina, Sicily, 114 cases were seen between the years 1928 and 1938,¹⁴ and this had increased to a total of 191 by 1948.¹⁵ In 1931, Auler and Wernicke¹⁶ reported that 1.4 per cent of 585 canine tumors collected in Germany were of this type. In contrast, the transmissible tumor has not been seen in Sweden¹⁷ and is apparently rare in Denmark.^{18, 19}

In the United States, there may be some variation in the incidence of this disease. For example, one observer²⁰ reported that the tumor is common in Texas (where 11 per cent of 100 consecutive canine tumors were of this type), whereas he had seldom seen it in Iowa. This neoplasm, apparently, is rare in Ohio,²¹ Kansas,²² Michigan,²³ and Minnesota. During the early part of the twentieth century, numerous cases were encountered in New York City, but now it is rarely seen there.²⁴ A similar decrease in frequency of occurrence has been noted in Philadelphia.²⁵ On the other hand, in a veterinary clinic in Evanston, Illinois, the transmissible venereal tumor constitutes 10 per cent of the tumor cases examined and is second only to mammary tumors as the most common tumor of dogs.²⁶

In males, the clinical picture is variable, depending on the extent of involvement of the penis, the presence of metastasis to the inguinal nodes and the age of the tumor. In early stages, there may be signs of irritation

such as serous or sanguineous discharge and licking of the affected parts. Examination of the glans penis reveals single or multiple small reddish firm nodules (FIGURE 1). As the tumors increase in size, they frequently bleed, ulcerate, and eventually undergo necrosis. Masses up to 5 cm. in diameter may be seen. The term "cauliflower growth" has been applied to the tumor.

In females, involvement of the vaginal mucosa is seen as small single or multiple reddish nodules, varying in diameter from several millimeters to large ulcerating irregular masses that protrude from the vulva. The natu-



FIGURE 1. Transmissible venereal tumor on the penis of an eight-year-old cocker spaniel. Two weeks before examination the owner noted enlargement of the left inguinal lymph node. The large mass at the end was firm and red, and measured 2 cm. in diameter. Two small, firm, red nodules (one of which is shown) were found 4 cm. posterior to the large tumor (Photograph courtesy of the School of Veterinary Medicine, University of Minnesota).

rally occurring disease is seen more often in females than in males^{12, 13, 27} and occurs most frequently during the years of greatest sexual activity. According to Wade,²⁸ Bashford, Murray, and Cramer²⁹ and Matsuba and Hiroye,⁸ the transmissible venereal tumor does not occur in virgin females. It is the observation of most veterinarians that the general health of affected animals is not impaired unless the tumors become necrotic and infected or occlude the urethral orifice.

Metastatic growths occur rarely and usually involve only the regional lymph nodes. In experimentally inoculated animals, metastatic growths to the internal organs have been seen.³⁰ Some workers deny that this tumor does metastasize.²⁷ Extragenital situations of this tumor are occasionally seen in the absence of genital involvement.^{15, 26, 31, 32} It is of interest that

the first reported experimental transmission of this tumor by Nowinsky³¹ in 1876 was done with material taken from the nose of a dog. It is not recorded whether or not this animal had also genital lesions. In view of the ease with which this tumor may be transferred experimentally to extragenital sites, it is not unlikely that the tumor may be transmitted from dog to dog through wounds.

One of the important features of this tumor is the ease with which it is transmitted from male to female and vice versa, during copulation. Hobday³³ recorded that in certain kennels it had worked such ravages that it became necessary to warn breeders of dogs against mating their animals until they had been carefully examined. Smith and Washbourn^{34, 35} recorded a situation in which one male was mated to 12 females, in 11 of which the tumor developed. A second male was subsequently mated with three of the infected females and acquired the tumor, and before it was recognized had transmitted it in turn to one of two females. White³⁶ observed transmission of the tumor by one male to six females. Two of these were successfully treated by surgical removal of the tumor, but the other four eventually died. In each of three males that had been mated with one of these females, the tumor developed.

Experimental transmission may be accomplished only by use of viable cells.^{6, 25, 30, 37, 38} In spite of many attempts, no etiologic agent separable from live cells has been demonstrated. Wehr,^{39, 40} Geissler,⁴¹ Smith and Washbourn⁴² and Sticker³⁻⁶ were among the first to make extensive studies on serial transplants of the transmissible tumor of dogs. Since then, a large number of investigators have shown that the tumor is readily transferred by either the subcutaneous, the intraperitoneal or the intravenous route. Sticker,⁴ von Dungern⁴³ and Wade²⁸ succeeded in transferring the transmissible venereal tumor of dogs to foxes, but other species including the common laboratory animals appear to have been refractory to all attempts to transmit the tumor experimentally.

A well-known and interesting feature of the transmissible venereal tumor of dogs is the apparent immunity that occurs following spontaneous regression or surgical removal. Sticker^{4, 6} mentioned that in his experiments he had many dogs which were resistant to successful implantation of the tumor after spontaneous regression of previously implanted tumor. Crile and Beebe⁴⁴ believed that the immunity may be humoral and made transfusions from dogs that had recovered spontaneously or were naturally immune into ten dogs bearing actively growing tumors. In all but three, there was complete or partial regression of the tumors. These authors believed that the regression was due to the transfer of some specific immune material. DeMonbreun and Goodpasture³⁰ believed that regression of experimentally implanted tumors was due to the establishment of an immunity, since it was not possible to reinoculate recovered animals successfully. They did succeed, however, in transmitting the tumor subcutaneously to animals already bearing large multiple tumor nodules. It was concluded that the immunity was variable and that it could be successfully overcome by injection of large amounts of cells. Of particular interest was the demon-

stration that serum from rabbits, into which emulsions of tumor cells had been injected, was capable of destroying tumor cells *in vitro*, whereas serum from rabbits immunized against normal dog tissue failed to do so.

Microscopically, the transmissible venereal tumor of dogs is composed of round, oval or polyhedral cells which, with the ordinary hematoxylin-eosin stain, have indistinct boundaries and poorly stained cytoplasm. The cytoplasm is faintly pink or may even be bluish. There are no granules or

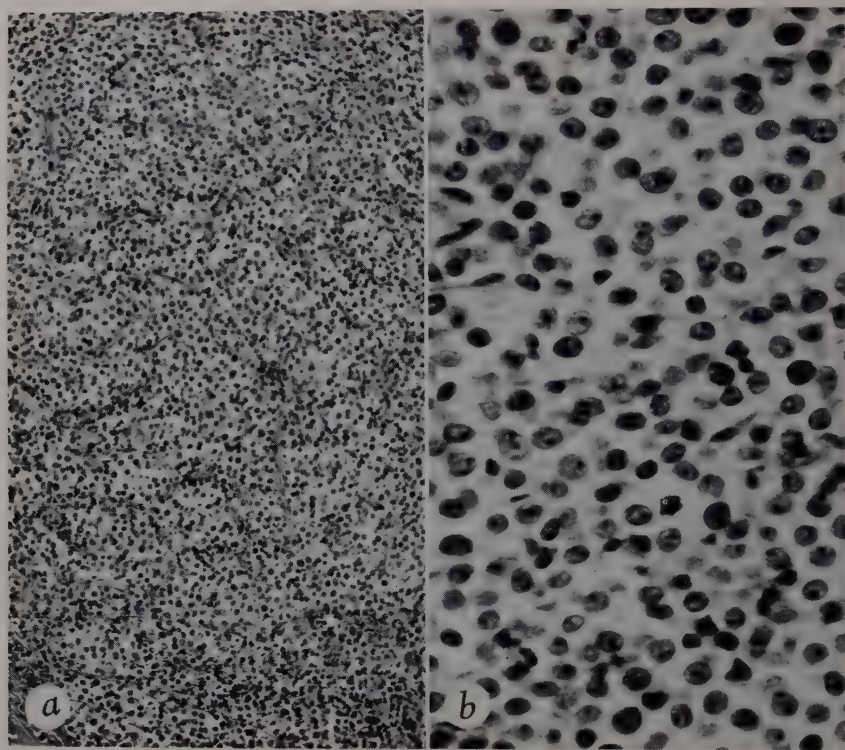


FIGURE 2a. Transmissible venereal tumor. This was removed ninety-seven days after implantation in the thirty-seventh generation. The arrangement of closely packed cells with a delicate stroma is also typical of the naturally occurring tumor. Most of the tumors in this transmission series had a similar microscopic appearance (hematoxylin and eosin 130X). 2b. Same as 2a, 540X.

cytoplasmic inclusions. The nucleus is large, round or oval with minute clumps of chromatin and a well-defined nucleolus (FIGURE 2b). Mitotic figures are common. Under low power, the sections usually appear as broad compact masses of cells with a faint connective tissue stroma that may suggest an alveolar arrangement (FIGURE 2a). Less commonly, the cells may be arranged in rows or cords with a well-defined stroma, as shown in FIGURES 3a and b, or there may be a loose structure with the cells arranged in single rows along delicate strands of stroma, as in FIGURES 4a and b. In older tumors and especially those with surface ulceration, there are

present inflammatory cells and extravasated blood. Large areas of necrosis are seen during stages of regression (FIGURE 5).

A few of the early investigators of the transmissible venereal tumor believed that the process was not actually neoplastic. Duplay and Cazin,⁴⁵ who made successful transplants, considered the process to be inflammatory; and Bashford, Murray and Cramer,²⁹ von Dungern,⁴³ and Wade²⁸ believed that the implanted cells stimulated the tissue of the host to give rise to

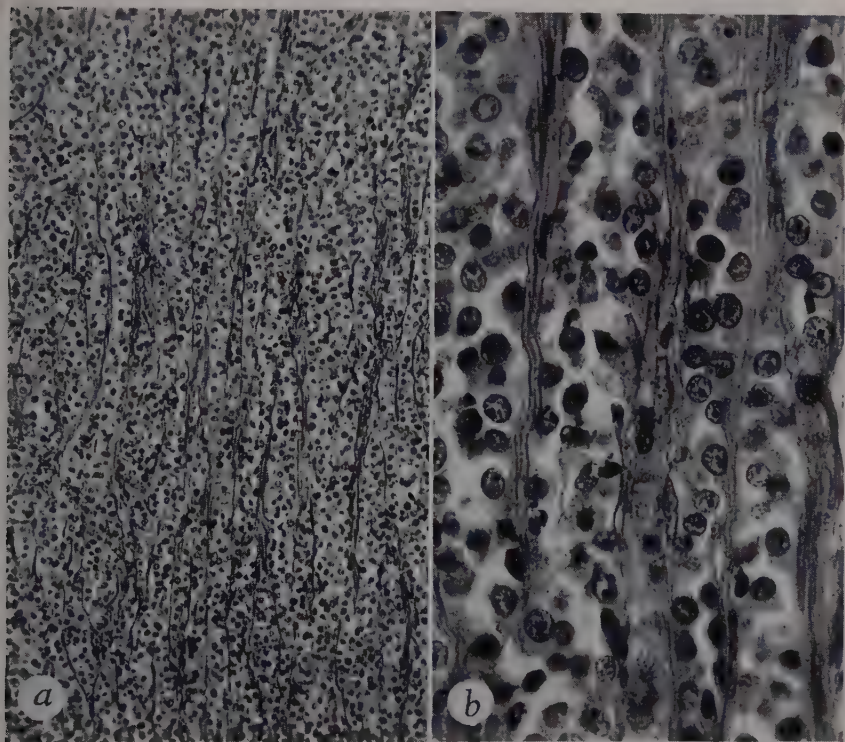


FIGURE 3a. Transmissible venereal tumor. This was removed fifty-three days after implantation in the twenty-second generation. Another section from this tumor presented a closely packed arrangement of cells with little stroma similar to FIGURE 2 (hematoxylin and eosin 130X). 3b. Same as 3a, 540X.

new tumors. Recently, Nanta and associates¹² have revived the idea that the process is inflammatory in nature. It is now, however, generally believed that Sticker,⁵ Beebe and Ewing,³⁷ and Hunter, Laws and Loeb³⁸ were correct in their interpretation that this tumor is a real neoplasm, that it arises by multiplication of the tumor cells, and that the tissues of the host do not take part in the new growth.

The origin and the nature of the cells are a matter of dispute. Feldman³² stated that the designation "lymphosarcoma" is made for morphologic reasons only and that the exact histogenesis is not conclusively determined. DeMonbreun and Goodpasture³⁰ expressed the belief that the origin of the

cells is not definitely determined and that they are probably derived from cells of the lymphocytic series. Stubbs and Furth,²⁵ however, can find no evidence that the cells are lymphocytic in origin. Mulligan² chooses to call the tumor a histiocytoma, but the evidence to suggest this designation is not convincing. Kaalund-Jørgensen and Thomsen¹⁸ think the cells resemble reticulum cells and therefore the tumor may be a reticulosarcoma.

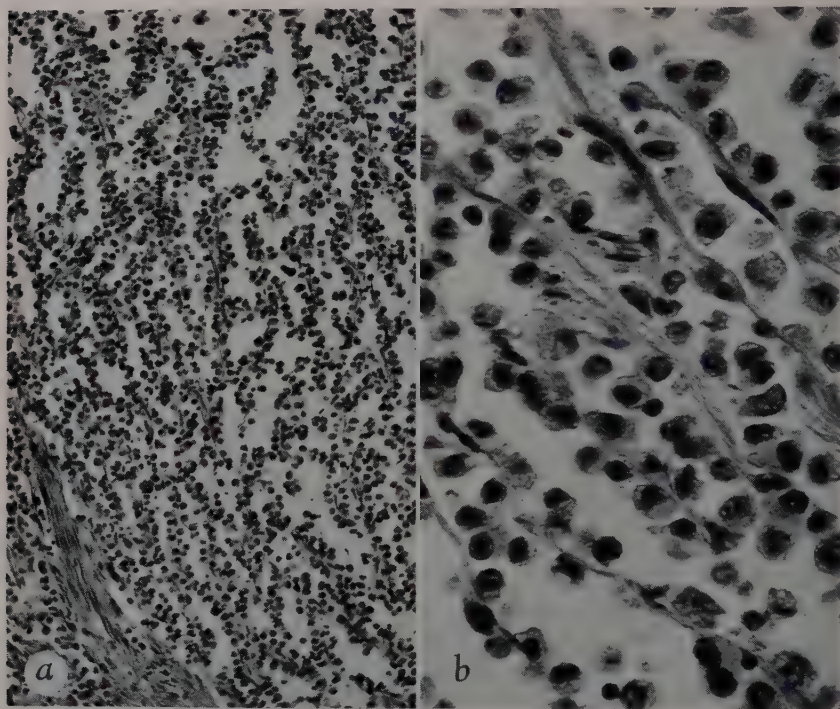


FIGURE 4a. Transmissible venereal tumor. This was another tumor removed from the same animal and at the same time as the tumor shown in FIGURE 3. This loose arrangement of cells attached to fine strands of stroma was encountered less frequently than that shown in FIGURE 3 (hematoxylin and eosin, 130X). (A similar structure is depicted for a naturally occurring case by Ajello¹⁴ and by Wade.²³) 4b. Same as 4a, 540X.

They state, however, that the tumor occupies a special place among malignant neoplasms and they prefer to use the term "transmissible venereal tumor of dogs" until more information is available. Bloom, Paff and Noback²⁴ believe that the cell is a "mature end cell of reticulo-endothelial origin", but recommend that the term "transmissible venereal tumor" be used. Jackson^{46, 47} observed that the cells of the heart base tumor in dogs and those of the transmissible venereal tumor are very similar and postulated that they may have the same origin. Since the heart base tumor was thought to arise from neuroblastic elements, this would, according to Jackson,^{46, 47} make the transmissible venereal tumor of dogs a neuroblastoma.

Experimental

In our laboratory the transmissible venereal tumor of dogs was passed through forty generations over a period of seven years.* The original purpose of the experiment was an attempt to produce and maintain a malignant disease in a large experimental animal in order to study the pathogenesis of cachexia, which often accompanies malignant diseases of man. After several generations, however, it was seen that the experimentally transmitted tumor usually pursued a benign course without cachexia and, furthermore, there was a high rate of regression and spontaneous

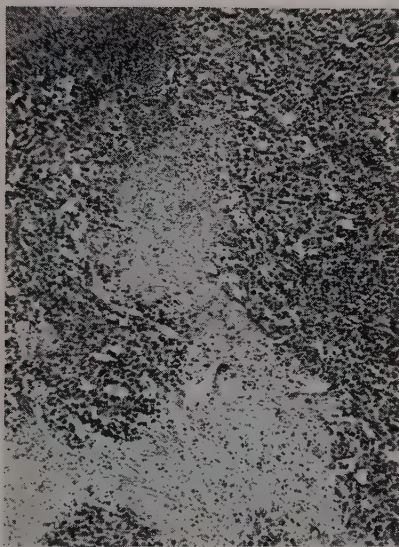


FIGURE 5. Transmissible venereal tumor undergoing degeneration (Hematoxylin and eosin, 100 \times . Tissue furnished through courtesy of Dr. Rue Jensen, School of Veterinary Medicine, Colorado Agricultural and Mechanical College, Fort Collins, Colorado).

recovery. Since the procedure of making implants was relatively simple and required little time, it was decided to continue the serial passage of the tumor to learn whether there would be any increase in its malignant propensities and whether there would be any histologic variation accompanying such changes.

Methods. Transplantations were accomplished as follows.⁴⁸ Using local anesthesia and careful sterile technic, a tumor was removed from the donor and immediately cut into slices about 1 mm thick. These were spread on sterile cork. The tip of a sharpened cannula with a diameter of 2 mm (FIGURE 6) was filled with tumor tissue by pressing it into the slice. Each transplant was thus in the shape of a disk about 1 mm thick and 2 mm in diameter. The recipients were prepared by passing a larger cannula (FIGURE

* This phase of the study was done by Dr. F. C. Mann.

6) with a sharp stilet into the subcutaneous tissue at the desired site of implantation. The stilet was removed and replaced by the narrow cannula containing the disk of tumor tissue in its tip. The blunt stilet of the narrow cannula was used to push the tumor tissue out into the subcutis of the recipient. By this method, it was easily possible to make 10 to 20 implantations per hour.

In most of the dogs, a transplant was made in the axilla and another transplant in the subcutis over the xiphoid cartilage. These areas could

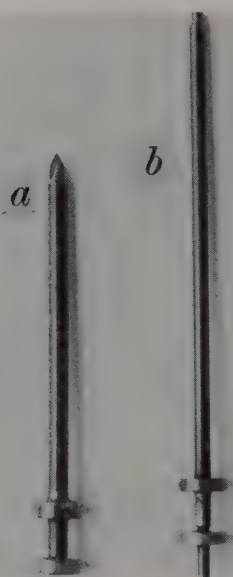


FIGURE 6. Cannulae used in transplantation of tumor. The cannula with the smaller diameter was filled with tumor tissue at the tip by pressing the sharpened end into thin slices of the tumor placed on sterile cork. The loaded cannula was then passed into the large cannula which had been previously inserted into the subcutaneous tissue of the recipient. The small plug of tumor tissue in the tip of the small cannula was forced into the tissue by means of the blunt stilet. Actual size. (Photograph obtained through the courtesy of Dr. W. H. Feldman.)

not be molested by the dog and also permitted ease and rapidity of subsequent palpation. The wounds healed readily. The recipients were examined approximately every two weeks until a tumor was palpable; then an examination was done every three or four weeks. In the case of negative animals, the biweekly examination was made for three or four months followed by monthly examinations.

The original donor was a mature female of mixed breed in a good state of health. When brought to the laboratory, the animal was found to have a "cauliflower-like" growth protruding from the vulva. A biopsy specimen presented the appearance of a transmissible venereal tumor. Only a part of the tumor was removed for preparing the transplants. Eighteen mature dogs served as recipients for this first generation. The original dog died suddenly from unknown causes five months later. The vaginal tumor was

still present and had not changed much in appearance. No other growths were found.

Tumors were subsequently found at one or both implantation sites in eight of the 18 dogs in the first generation. Three of these were selected as donors for three respective groups of 10 dogs each, which constituted the second generation. During the following seven years, the tumor was transferred through forty generations. Five of the generations consisted of three groups of recipients, as in the second generation. Twelve generations consisted of two groups and the remaining twenty-three generations had only one group. There were, in all, sixty-two groups of nine to ten

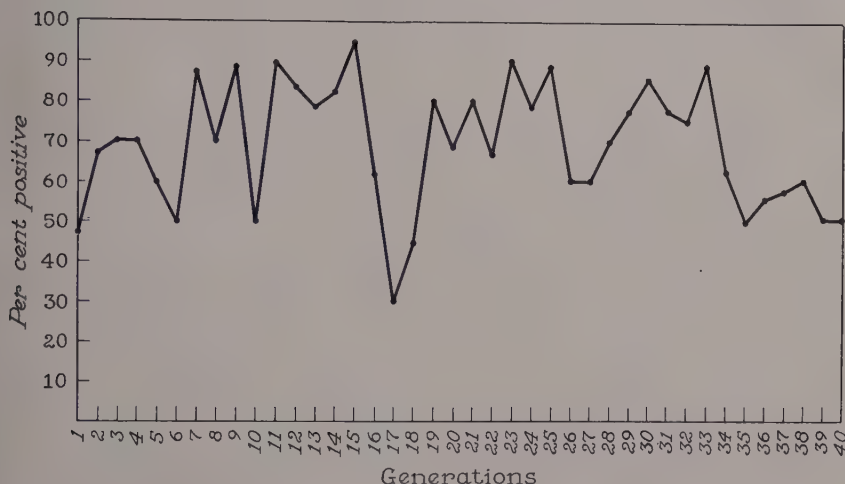


FIGURE 7. Percentage of positive results in each of the forty generations. The first seventeen generations contained 20 to 30 dogs each. The subsequent generations consisted of 9 or 10 dogs each. The fifteenth generation with 95 per cent positive results consisted of 20 males, as did the seventeenth which had 30 per cent, the lowest percentage of positive takes. There is no evidence that the "virulence" of the tumor was either diminished or augmented during the forty passages over a period of seven years.

dogs each for a total of 601 dogs. Tissues from each of the donors in each generation were preserved in ten per cent formalin solution. These were stained with hematoxylin and eosin. A few were stained by Gömöri's⁴⁹ method for reticulum fibers.

All the animals were mature healthy individuals of mixed breed. No very young or very old animals were used. The range in age was estimated to be two to six years. Sixty-five per cent were males and 35 per cent females. In a few generations, all the animals were males but, in general, the ratio was three or four females to six or seven males in each generation.

Results. A positive result was recorded for those dogs in which there developed a palpable nodule at the site of the implantation. When first detected, the nodules were about 5 mm in diameter. As will be discussed later, the majority grew slowly, reached a size 2 to 3 cm in diameter, then regressed without ulceration of the overlying skin. Of the 601 dogs, only 37 (6 per cent) were considered to be failures because of premature death.

These died within three weeks after implantation from distemper or unknown causes and are excluded from the data.

Of the remaining 564 dogs, in 385 (68 per cent) tumors developed. In most of the dogs, a tumor was detected at both sites. In those in which a growth could be detected at only one site, it occurred with equal frequency either in the axilla or over the xiphoid cartilage.

There was some wide variation in the number of positive results in each generation, as indicated in FIGURE 7. The highest percentage occurred in the fifteenth generation, in which tumors developed in 19 (95 per cent) of 20 dogs (all males), and the lowest occurred in the seventeenth, in which only six (30 per cent) of 20 dogs (all males) were positive. Although, with one exception, all the generations after the first had a higher percentage

TABLE 1

TRANSMISSIBLE VENEREAL TUMOR OF DOGS. FIRST APPEARANCE OF TUMOR DETECTABLE BY PALPATION AT SITE OF IMPLANTATION IN 381 DOGS

<i>Day of first appearance</i>	<i>Number</i>	<i>Per cent</i>
15	2	0.5
20-30	139	36.5
31-40	64	16.8
41-50	71	18.6
51-60	62	16.3
Subtotal	338	88.7
61-70	19	5.0
71-80	6	1.6
81-90	9	2.4
91-134	9	2.4
Subtotal	43	11.3
Total	381	100.0

of positive results than the first one, the differences are not great and also the variation between generations is too wide to permit any conclusion regarding a change in the ability of the tumor to become established.

First Appearance of Tumor. In most of the dogs, the two sites of implantation presented palpable growths at approximately the same time. There were some differences, however. In one animal, for example, the growth in the axillary space was detected in thirty-four days, while that over the xiphoid was not observed until seventy-three days later. In another, the transplant over the xiphoid was seen in twenty-eight days, while that in the axilla did not appear until fifty-two days later. The location seemed to have no influence on the time required for the tumor to become evident.

The date of the first appearance of the tumor at either site was not recorded for four animals. Referring to TABLE 1, it is seen that the "incubation" time was variable but within some general limit. In 88.7 per cent, the tumors were seen within the first sixty days. More than half were palpable within the first forty days. In only a few animals was there a

prolonged period before there was palpable evidence of growth. One animal had been declared negative ninety days after implantation but, when examined at necropsy on the one hundred and thirty-fourth day, small tumors were found at both sites, which histologically appeared to be viable.

Negative Animals. One hundred and seventy-nine (32 per cent) of the 564 dogs failed to present palpable growths at either site of implantation and were declared negative after 90 to 120 days. With the exception noted in the previous paragraph, none were known to have tumors after

TABLE 2

TRANSMISSIBLE VENEREAL TUMOR OF DOGS. DURATION OF EXPERIMENTALLY IMPLANTED TUMOR. THE TIME WAS MEASURED FROM THE FIRST APPEARANCE OF THE TUMOR UNTIL IT WAS NO LONGER PRESENT AS DETERMINED BY PALPATION. 328 DOGS

<i>Days</i>	<i>Number</i>	<i>Per cent</i>
20	4	1
21-40	52	16
41-60	81	25
61-80	40	12
Subtotal	177	54
81-100	23	7
101-120	33	10
121-140	22	7
141-160	19	6
161-180	11	3
Subtotal	108	33
<i>Months</i>		
6-7	15	4.6
8-9	11	3.4
10-11	7	2.1
12-18	5*	1.5
19-24	1†	0.3
30-36	3†	0.9
52	1†	0.3
Total	328	

* Three animals killed at 15, 17 and 18 months respectively after appearance of tumor. No evidence of regression.

† Killed before tumor disappeared.

that period. Eighty-one of these animals were subsequently examined at necropsy twelve to thirty-six months after implantation of the tumor and nothing could be seen at the site of the implant.

Duration of Tumor. The length of survival of the tumors is known for 328 positive dogs. The remainder either died before their tumor regressed or else the date of disappearance was not known. As shown in TABLE 2, there was a high rate of spontaneous regression. Forty-two per cent of the dogs did not retain palpable growths at the site of implantation longer than sixty days. More than half (54 per cent) of the tumors had regressed by eighty days and 87 per cent of the positive dogs failed to have detectable growths six months after the first appearance.

With very few exceptions, the tumor regressed without superficial evidence of necrosis. There was usually no ulceration and sloughing of the overlying skin. Most of the growths were about 5 mm in diameter when first observed. They were firm, rounded and loosely attached in the subcutis. They grew slowly. Those that persisted sixty days or longer measured approximately 2 to 4 cm in diameter before disappearance. Some of those that were still present in six to ten months were 3 to 5 cm wide and several centimeters thick. The large tumors occasionally became softer than normal and disappeared over a period of several months. The smaller tumors disappeared relatively rapidly, in two to four weeks. In only 22 (7 per cent) of the entire group, did ulceration of the skin and necrosis of the underlying tumor develop. These animals were killed before the area healed. The last animal listed in TABLE 2 maintained tumors at both sites which gradually enlarged for four years until the one in the axilla extended posterior to the olecranon. It measured 10 by 7 by 3 cm. The tumor over the sternum measured 10 by 5 by 4 cm. After the forty-ninth month, the tumors began to soften, the skin sloughed off in patches and there was an offensive odor. Until the forty-ninth month, the animal appeared to be in good health and was not suffering any apparent ill-effects of the tumor until it became necrotic. The animal was killed fifty-two months after the first appearance of the tumor. No metastatic growths were found at necropsy.

There was no relationship between the time required for a tumor to appear and the survival time.

Metastatic Growths. Only five animals of the entire group had any evidences of metastatic growths. In three, the metastatic growths were limited to the prescapular lymph nodes which became enlarged and firm. These regressed along with the primary growth at the site of the implant. In one animal, there were two metastatic growths or secondary implants along the thorax opposite the side where the axillary implant was growing. This animal died, and inadvertently no necropsy was performed. In the other animal, there were small growths at both sites thirty days after implantation. These continued to increase in size until they measured 1.5 cm in diameter. Five months after the implants were detectable, metastatic growths were noticed on the back and on the head. These grew rapidly. Three months later, the metastatic growths on the back and head began to ulcerate and the animal was killed. Three tumors measuring about 7 cm each were found on the back. The tumor on the head measured 10 by 10 by 3 cm and appeared to be invading the right socket. There were also metastatic nodules in both parotid glands measuring 2 by 2 by 2 cm. No metastatic growths were found elsewhere.

No recurrences were observed in any animal in which the tumors had regressed spontaneously. Fifty-six dogs were observed for periods of one to three years. Nine dogs were kept for four to five years and one was maintained for ten years without any evidence of tumors developing where previous tumors had regressed. Ninety-four animals were observed for periods of 100 days to one year, and none of these were found to have recurrences of their tumor in that period.

Microscopic Appearance. There was no change in the microscopic structure of the tumor during the entire series. Most frequently, it appeared as shown in FIGURE 2a and b but variation in the amount of stroma altered this uniform pattern to produce an arrangement of cells as shown in FIGURES 3 and 4. Material for transplants was taken from donors when their tumors were 30 to 60 days old and no microscopic evidence of regression was found in these. No intercellular reticulum fibrils were seen in any of the sections stained by the method of Gömöri.⁴⁹ Specimens obtained at necropsy from ulcerating and necrotic material showed tumor tissue containing lymphocytes, polymorphonuclear leukocytes, tissue debris and erythrocytes.

Fate of Donors. Data are available on 57 of the 62 donors. In seven of them, only a portion of one tumor was removed. In three of the seven, the remaining portion diminished in size and disappeared in about thirty days. Unfortunately, no record was made of the fate of the other tumor in these seven dogs. In the others, the growth remained more or less stationary in size and eventually disappeared, in periods ranging from 150 to 240 days.

In the remaining 50 donors, the entire tumor at both sites was removed to furnish transplants but no definite attempt was made to insure that all the neoplastic tissue was removed. In this respect, the surgical excision differed from one that would be done for therapeutic purposes. The tumor recurred at one or both sites in 14 of the 50 dogs. The earliest recurrence was noted sixteen days after operation and the latest was seen in seventy-nine days. The others were seen in thirty to fifty days. In one animal, there was a recurrence 50 days after operation. The tumor slowly grew and, in 120 days, it was about 10 cm long and 4 cm wide, at which time it became soft, ulcerated and foul-smelling. The animal was killed one month later. With the foregoing exception, all the recurrent tumors eventually regressed and completely disappeared within periods ranging from 30 to 240 days.

The animals in which no recurrences were noted were observed for periods ranging from 101 to 240 days before being declared free of the neoplasm.

Comment

It is fruitless, perhaps, to speculate on the reasons for the apparent marked differences in the geographical incidence of transmissible venereal tumor of dogs. The first reaction to this peculiarity of the disease is that it is not being recognized where it does exist. Investigators who are familiar with the clinical and microscopic characteristics of the disease, however, are at least aware of the rarity of the neoplasm in their localities, as mentioned previously.^{20, 24, 25} In our laboratory, with the exception of the original donor for the experiment reported in this paper, no naturally occurring transmissible venereal tumor has been encountered among hundreds of dogs in twenty-five years, except three animals in our kennels, in which the neoplasm developed during the period when they had the opportunity of associating with animals bearing the experimentally transplanted tumors. We believe that if the tumor were as common in our locality

(Rochester, Minnesota) as it is reported to be elsewhere, we would have recognized it.

Certain questions are raised which cannot be answered with information now available. How is this tumor maintained where it is common? Are there reservoirs other than dogs? Are there more ownerless dogs that roam freely in areas where the tumor is frequently seen as compared to regions where the tumor is not known? Are there differences in susceptibility between breeds of dogs?

In the experiment reported in this paper, one is impressed with the fact that 32 per cent of the animals were apparently resistant to successful implantation of the tumor. Since all were mature animals, it could be said that they were immune as a result of previous natural exposure and spontaneous recovery. If this is true, then the tumor is not as rare in our locality as we suppose. It would be instructive in this regard to test the susceptibility of dogs before the age of puberty and of mature females and males who were castrated before puberty.

The high rate of regression of the experimentally transmitted tumor is of particular importance, especially with respect to the use of this neoplasm in studying chemotherapy of experimental malignant disease. The various types of therapy reported to cause regression of the lesions may be open to question. Nanta and associates¹² believed that the administration of nicotinamide and also roentgen therapy were responsible for cures in their studies. The rate of recovery in their therapeutic trials, however, was not appreciably greater than the rate of spontaneous regression seen in our series. The same may be said of the results reported for the use of radium and surgical treatment by Wong and K'ang.⁹ The lack of adequate controls and the use of relatively few animals permit one to doubt the therapeutic value of such varied agents as bacterial toxins,⁵⁰ liver extracts,⁵¹ sodium arsanilate (atoxyl) and also foreign protein,⁵² all of which have been reported to cause disappearance of transmissible venereal tumors of dogs.

We agree with Lesbouyries²⁷ that it is prudent to avoid an attempt to classify this tumor. The cell type is a matter of dispute. It appears not to arise from the tissues of the host, which makes it impossible to study the histogenesis *in vivo*. The neoplasm is transmitted from dog to dog like a parasite and is maintained by the host until some immune mechanism of the host causes the tumor to disappear or, more rarely, until the tumor becomes so large that it interferes with the health of the host.

Until there is information based on conclusive evidence of the nature of this neoplasm, we believe that it is best designated as the "transmissible venereal tumor of dogs."²⁴

Summary and Conclusions

A review of the papers of historical interest and those which have contributed significantly to the present-day knowledge of the transmissible venereal tumor of dogs may be summarized as follows: The tumor is a true neoplasm, which usually occurs on the genitalia of both sexes and is

ordinarily spread by coitus. More females than males acquire the tumor naturally. The greatest incidence occurs in the age groups from one to six years, which is the period of greatest sexual activity. Extragenital occurrence of the tumor in the absence of genital involvement is recognized and is due possibly to transmission *via* wounds and abrasions. Metastatic growths are rare but do occur. This tumor has been encountered in many parts of the world but there are apparent variations in its incidence. It is common in some countries and rare in others. In the United States, there is also great geographical variation in the frequency of transmissible tumor of dogs.

The tumor is transmitted experimentally by transplantation of living cells only. No microbial or viral agent has been demonstrated. It may be implanted artificially by injecting viable cells *via* the subcutaneous, intraperitoneal and intravenous routes. Besides dogs, only foxes have been successfully inoculated. The naturally occurring tumor and those established by artificial transfer tend to regress. Dogs in which tumors have regressed spontaneously or have been removed surgically are resistant to further successful transmission. In spite of the malignant microscopic appearance of the tumor it is clinically benign in most instances. In some animals, however, the growth may steadily increase in size, become ulcerated, infected and may even metastasize to adjacent lymph nodes or, rarely, to internal organs.

Microscopically, the tumor consists of large rounded cells with indistinct cytoplasm, a large vesicular nucleus and a prominent nucleolus. The presence of many mitotic figures is characteristic. The stroma varies in amount from thin delicate strands, giving the tumor an alveolar appearance, to broad bands, giving rise to a cordlike arrangement of the cells. The nature and origin of the cells are not known. Various attempts to classify the tumor as lymphosarcoma, reticulosarcoma, histiocytoma, and neuroblastoma are inconclusive.

An experiment is described in which the tumor was passed through forty generations using sixty-two groups of recipients for a total of 601 dogs. Only 37 (6 per cent) of these were failures due to premature deaths; 385 (68 per cent) of the remaining 564 dogs developed palpable tumors at the site of implantation. The percentage of positive animals varied widely from generation to generation, the lowest being 30 per cent and the highest, 95 per cent. The earliest appearance of tumors was recorded in two dogs fifteen days after transplantation. Thirty-seven per cent had palpable tumors at the site of implantation within thirty days and, by the end of sixty days, 88.7 per cent were detectable. The rate of regression and disappearance of the transplanted tumors was high. Seventeen per cent lasted less than forty-one days. More than half of the positive dogs experienced spontaneous recovery within eighty days. Only 13 per cent of the positive dogs retained their tumor longer than six months.

The variable percentage of successful transplants and the high incidence of spontaneous regression of the experimentally transmitted tumor detracts from usefulness of this neoplasm for studies in experimental therapy.

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ELECTRON MICROSCOPY OF VIRUSES OF HUMAN PAPILLOMA, MOLLUSCUM CONTAGIOSUM, AND VACCINIA, INCLUDING OBSERVATIONS ON THE FORMATION OF VIRUS WITHIN THE CELL*

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This discussion will deal with electron microscopic studies of two lesions of human skin, papilloma and molluscum contagiosum. Both diseases are infectious, the etiological agents being filterable. Work on these viruses has been limited, however, in that the only known host is man. Our work on human papilloma was carried out in conventional fashion, *i.e.*, isolation of cellular components by differential centrifugation and their examination in the electron microscope. Molluscum contagiosum was studied in the same way, but, in addition, infected cells have been cut in thin sections and the virus examined *in situ*. For purposes of comparison, thin sections of vaccinia-virus-infected cells of the chorio-allantoic membrane have been studied. It will soon become obvious that two pox viruses do not necessarily multiply in the same fashion.

Human Papilloma Virus

The viral etiology of warts was established over 30 years ago by the production of verrucae on human skin by inoculation of a filtrate of wart suspension.¹ Supporting evidence was supplied by our recent observations^{2,3} of crystalline virus-like particles obtained from papillomatous growths removed from human skin. The papillomas or verrucae from which these elementary bodies are obtained show a similar histopathologic picture, differing from the usual verrucae in that intranuclear inclusion bodies are found in the rete cells and also cytoplasmic masses in the same layer of the epidermis. It is relatively simple to obtain elementary bodies from the human lesion.

Material from the papillomas and from control specimens is ground with alundum and distilled water, centrifuged at 2,000 r.p.m. for five minutes, and the resulting supernatant fluid subjected to further centrifugation at 6,000 r.p.m. for 45 minutes. The sediment is resuspended in a small volume of distilled water (about 1 ml.). For electron microscopy a small drop is placed on a collodion screen and shadow-cast with chromium or palladium before examination. Although isolated particles may be found in all specimens, the particles are arranged in crystalline-like clusters with such regularity that this arrangement appears to be characteristic. The particles are spherical and, when in crystalline array, average 52 m μ in diameter, with a range of 50 to 54 m μ . When these particles are not in crystalline array, they average 68 m μ in diameter with a range of 56 to

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80 μ . Typical illustrations are shown in FIGURES 1 to 8. The papilloma virus particles are morphologically stable when stored in distilled water at 4°C. for 10 months, and they have been recovered from tissue stored in the frozen state for one month. Preparations from other warts (without intra-

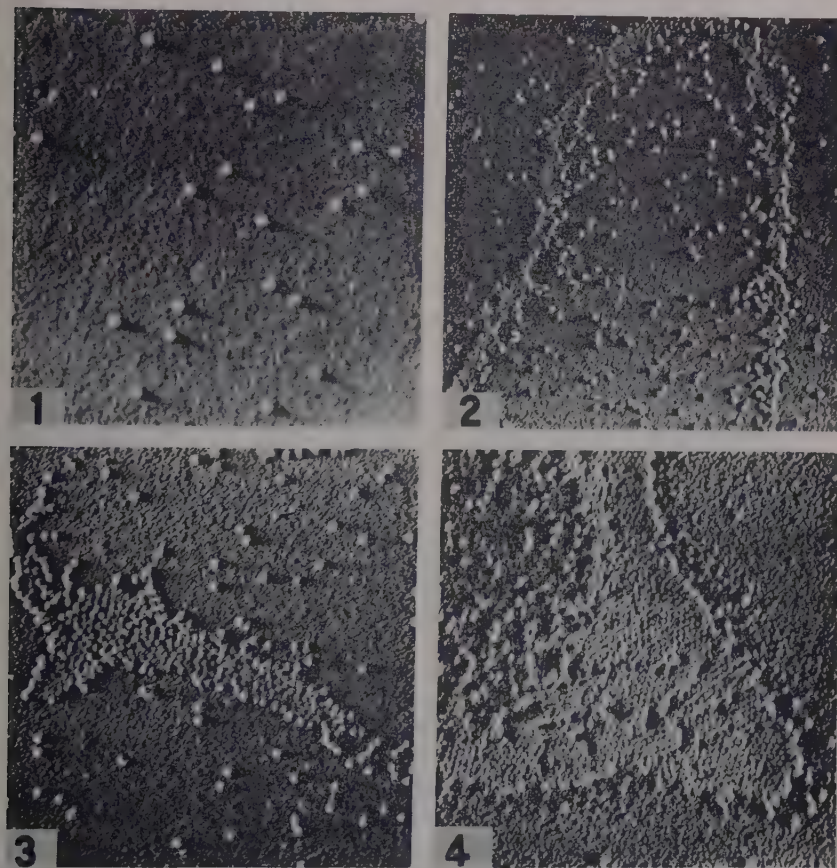


FIGURE 1. Spherical elementary bodies (virus particles) isolated from human papilloma by differential sedimentation. 16,500 x

FIGURE 2. Early stage of aggregation of particles. 11,000 x

FIGURE 3. Beginning of orderly arrangement of particles. 15,000 x

FIGURE 4. Two-dimensional crystalline arrangement of particles. 12,500 x

nuclear inclusion bodies) and from normal human skin have revealed no uniform particles, but only amorphous scattered clumps of matter, collagen fibres, and spherical particles of varying diameter.

The formations of virus particles are generally one layer thick, having the appearance of a two-dimensional crystal. At times, however, the particles are piled in successive layers and the continued regularity in three-dimensions is, by definition, a true crystal. If the particles are true spheres packed tightly in a hexagonal arrangement, connecting particles in

adjacent rows should make angles of 120° with each other, and this has been observed in our preparations.

Histopathology. The histological appearance of the papillomas yielding virus-like particles is described in detail in a paper by Bunting and Strauss.⁴ They consisted of irregular upgrowths of thickened epidermis overlying

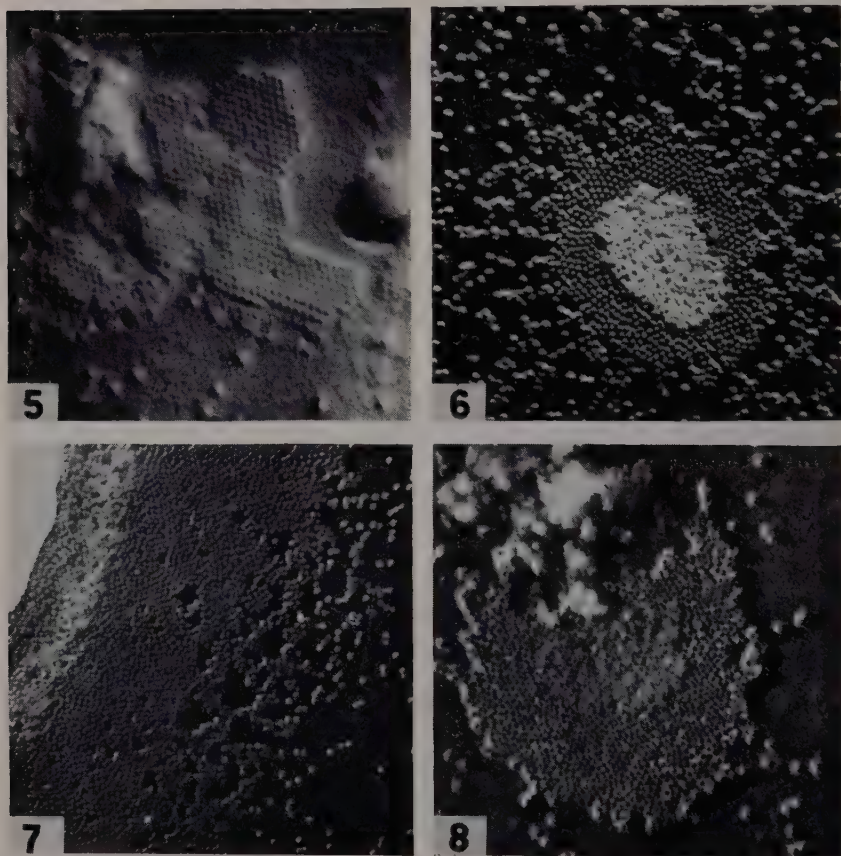


FIGURE 5. Crystalline formation of particles several layers deep and having uniform structure in three dimensions. 19,000 x

FIGURE 6. Double layer of particles. 15,000 x

FIGURE 7. Orderly array of particles, part of which is multi-layered. 10,500 x

FIGURE 8. Orderly array of particles from a preparation held in aqueous suspension at 4° for 10 months.

elongated papillae containing fine blood vessels and delicate connective tissue. There was a great increase in the thickness of the stratum corneum with persistence of pyknotic nuclei in the lower layers, where the keratinized substance was loosely arranged and not as compact as at the surface. Significantly, there were intranuclear inclusion bodies and characteristic cytoplasmic masses in most of the cells of the papillomas. These changes were lacking in the basal cells, but were present in the first or second layer

of cells above them and in all of the more superficial layers. There was one intranuclear inclusion body in a nucleus. It was round, eosinophilic, and frequently surrounded by a very narrow halo in the fixed section. These bodies averaged $2.4\text{ }\mu\mu$ in diameter ranging from 1.0 to $3.2\text{ }\mu\mu$. They were smallest when they first appeared in the lower Malpighian layers and increased in size progressively until the stratum corneum, where they could not be identified within the heavily stained pyknotic nuclei.

All of the cells that contained intranuclear inclusion bodies also had cytoplasmic masses that exhibited a development paralleling the differentiation of the cells into their final state in the stratum corneum. The masses first were small and solid, or contained a single, tiny, clear vacuole. They were multiple, 10 to 20 in each cell. The granules were fewer but larger in the cells higher up in the Malpighian layers, at times almost reaching the size of the nucleus. The vacuoles within them also increased in size commensurately. The granules, at first, were faintly stained by hematoxylin but, as they enlarged, this staining tendency disappeared and they developed an affinity for eosin which became very strong in the lower layers of the stratum corneum. Here, the vacuoles disappeared and the granules apparently became fused into one or two solid masses occupying almost the entire cytoplasm.

As stated, these changes were not encountered in the lowermost cells of the Malpighian stratum. It is of interest that the latter cells were in no way different from corresponding cells in an unaffected region. The cells with inclusions were larger and apparently lacked intercellular bridges, although when one of them adjoined a normal-appearing cell, intercellular bridges were found. Abnormalities in nuclear structure have been observed. They were larger, heavily stained and irregular in outline. In one instance, there were four atypical small nuclei present in a single cell. Mitoses were never seen in these cells, although they were not infrequent among cells in corresponding position in uninvolved epidermis.

Histochemical studies have been carried out by one of us (H.B.) and the results of these have been presented elsewhere.²⁻⁴ It is noteworthy that the intranuclear as well as the cytoplasmic inclusions exhibit a weak basophilia, which is unaffected by ribonuclease. This is in contrast to the cytoplasmic basophilia of the lower layers of the adjacent epidermis which may be readily removed by this enzyme.

The Feulgen reaction for desoxyribonucleic acid was not given by either the intranuclear inclusion body or the cytoplasmic masses, while the nuclear membrane and chromatin material were strongly positive. Absolute identification of the intranuclear inclusion bodies in the Feulgen preparations, to avoid confusion with chromatin masses, was insured by comparison with drawings and kodachromes of the same cells when previously stained with hematoxylin and eosin. Photomicrographs were taken at wave lengths of 2650 and $2800\text{ }\text{\AA}$ and evaluated visually.* The intranuclear inclusion body showed about equal absorption to that of the general background

* The authors are grateful to Dr. Jesse F. Scott of the Department of Biology, Massachusetts Institute of Technology, and the Huntington Laboratory, Massachusetts General Hospital, for the ultraviolet photomicrography.

proteins at 2650 Å, while the nuclear membrane and chromatin exhibited greater absorption. At 2800 Å, background proteins, intranuclear inclusion body, and nuclear structures all appeared to have the same density. The cytoplasmic masses behaved similarly to the inclusion body.

The formations isolated from these papillomas constitute the first crystalline substance of this type obtained from any animal growth. This characteristic particle is probably virus in nature and responsible for the appearance and growth of the wart. In the past, spherical viruses have been found to yield such crystalline patterns. This has been particularly true of certain plant viruses and, on occasion, highly purified preparations of spherical bacteriophage have yielded similar patterns.⁵ However, the fact that relatively impure preparations of suspensions of these warts yield such patterns indicates the marked degree of attraction which these particles have for each other and their uniformity. The diameter of these particles, 52 mμ, is the same order of magnitude as that found for the virus of the Shope rabbit papilloma.⁶ It is a much simpler and more uniform substance than the elaborate elementary body which causes the skin lesion of molluscum contagiosum. Furthermore, the uniformity of particle size of this papilloma virus is in striking contrast to the variation which one finds among molluscum contagiosum virus particles. This latter variation is similar to that which one has come to expect in microbiological organisms.

The characteristic histopathological changes observed in these lesions, not seen in every common wart, are apparently associated with the presence of the virus-like particles described above. One would conclude that the normal development of the epidermal cells had been distorted to a greater degree than that occurring in simple hyperplasia. The eosinophilic intranuclear inclusion body presumably indicative of the presence of the virus, is small when it is first seen in the first or second layer of cells above the basal. It doubles in size by the time its identity is lost in the pyknotic nuclei of the parakeratotic stratum corneum.

The cytoplasm contains vacuolated granules that develop as the cell matures and finally fuse into masses in the stratum corneum. The information from histochemical studies is currently incomplete, but there is no evidence from ultraviolet photomicrography or Feulgen preparations of detectable nucleic acids in the intranuclear inclusion body. The nature of the cytoplasmic masses has not been determined. Ribonucleic acid was not identified.

It may be of interest to mention the incidence of eosinophilic intranuclear inclusion bodies and cytoplasmic masses in 156 verrucae vulgaris and plantaris removed consecutively in Dr. Strauss' practice.¹⁴ Of the 156 verrucae, 21 (13 per cent) showed such inclusion bodies and cytoplasmic masses. The greater number of these were found in verrucae plantaris as compared with verrucae vulgaris, the figures being 16 out of 37 plantar warts removed from 30 patients, or 43 per cent; and 5 out of 119 common warts removed from 75 patients, or 4 per cent. The greater frequency of positive findings in plantar warts may well be explained by the duration of the lesion. Plantar warts, by virtue of their location, cause discomfort

and inconvenience earlier than lesions situated elsewhere, and therefore will cause the patient to seek their removal at an earlier time. In this series of 156 lesions, the average duration of the lesion at the time of removal was five months in plantar verrucae and nine months in common verrucae.

Molluscum Contagiosum Virus

We turn now to another virus-induced lesion of human skin, molluscum contagiosum.⁷ Because this virus belongs in the pox group, we shall include for comparative purposes some observations made by the same method on the intracellular growth of vaccinia virus. Both viruses have been studied sufficiently in the purified state so that we can readily recognize them when seen within infected cells. Although both of these viruses produce inclusion bodies within the cytoplasm, they appear to multiply in different fashion.

By conventional means of preparation, which depend chiefly upon differential centrifugation of suspensions of infected tissue, one may readily obtain pure preparations of the virus of molluscum contagiosum (FIGURE 9). As several workers have observed, this virus is more or less brick-shaped measuring on the average, 330 by 230 m μ .

The morphology of molluscum contagiosum has been studied extensively under the light microscope. The lesion usually described consists of a localized region of the epidermis in which the cells are altered by the presence of cytoplasmic inclusion bodies that appear first in the Malpighian layer and reach maturity in the lower stratum corneum. Van Rooyen⁸ has demonstrated by microdissection that the mature globular inclusion body is surrounded by a sheath and is filled with elementary bodies lying in a gelatinous matrix. Goodpasture⁹ pointed out an enlargement of the nucleoli of early infected cells. Rake and Blank,¹⁰ in tracing the development of the inclusion body, maintained that there is, first, a general increase in cytoplasmic ribonucleic acid, which is followed by the appearance of diffusely scattered islands of desoxyribonucleic acid. The latter enlarged to form the characteristic inclusion body within which they described the ribonucleic acid compressed into septa.

The present study, which is reported in greater detail elsewhere,⁷ extends the observations on the morphology and stages in the development of the virus by the examination of thin sections of molluscum lesions with the electron microscope. Lesions of molluscum contagiosum freshly removed with a curette were fixed in 4 per cent buffered formalin for two to three hours. Pieces of tissue no larger than 1 to 2 mm. on an edge were dehydrated in a graded series of alcohols and embedded in methacrylate (1 part methyl and 2 parts butyl), using 2-4 dichloro-benzoyl peroxide as a catalyst, and incubated for 18 to 24 hours at 60°C. Sections were cut from 0.2 to 0.5 μ with a glass knife, mounted on collodion-covered grids, and, after removal of the methacrylate by xylol, were shadowed with palladium at an angle of approximately 1:7 before examination with an RCA electron microscope, model EMU.

In the prickle-cell layer of the epidermis of human skin taken at the site

of the lesion, cells infected with virus may readily be found. FIGURE 10 shows a mature inclusion body in a cell. The nucleus is shrunken and displaced to one side. Inclusion bodies may be divided into compartments

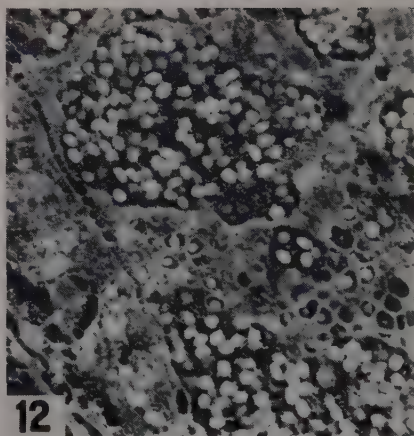
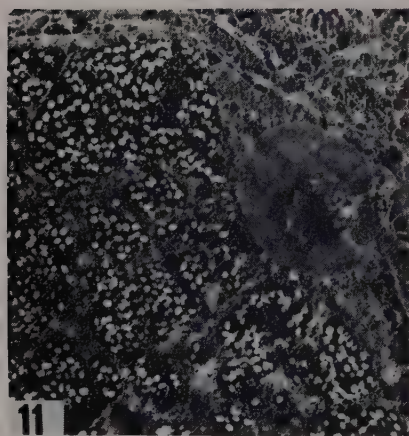
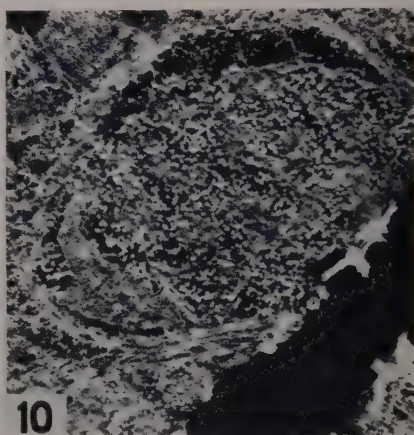
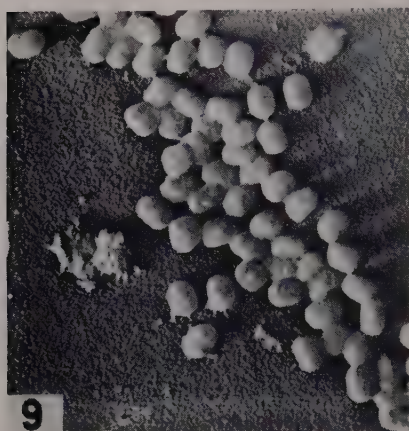


FIGURE 9. Pox-like elementary bodies (virus particles) isolated from molluscum contagiosum lesion of human skin. 15,000 x

FIGURE 10. Mature inclusion body almost fills the cell. Nucleus is shrunken and displaced to lower left. 2,000 x

FIGURE 11. Subdivision of inclusion body into nests of mature virus particles separated by thin walls. An enlarged nucleolus is present in the shrunken nucleus. 3,500 x

FIGURE 12. Part of an inclusion body, showing discrete, mature virus particles contained in locules. The cytoplasmic matrix surrounding these locules is being converted into virus particles. 5,500 x

by extremely thin walls, with mature virus particles filling the locules between the septa (FIGURE 11). In such infected cells the nucleolus may be greatly enlarged. FIGURE 12 shows part of an inclusion body at higher power. One can see that the locules contain discrete virus particles. The matrix of the cytoplasm surrounding these locules appears to be undergoing segmentation. As the virus particles are formed, they leave behind a cytoplasm which appears to be moth-eaten.

FIGURE 13 shows what we believe to be provirus, *i.e.*, the immediate precursor of the mature virus particle. Areas of the matrix composing the septa surrounding the mature virus particles appear to be cut out into rounded structures. These bodies are distinctly contoured and separated from the surrounding matrix by a more electron-translucent zone.

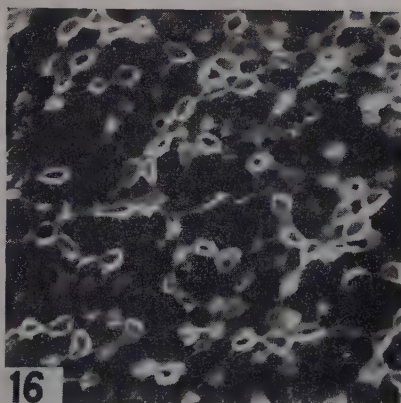
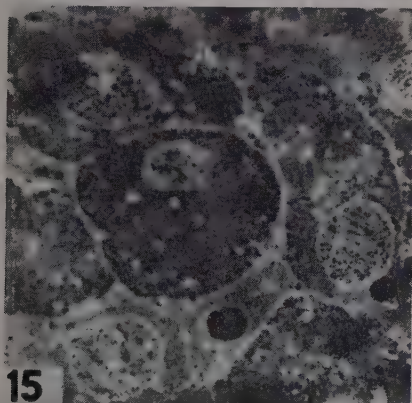
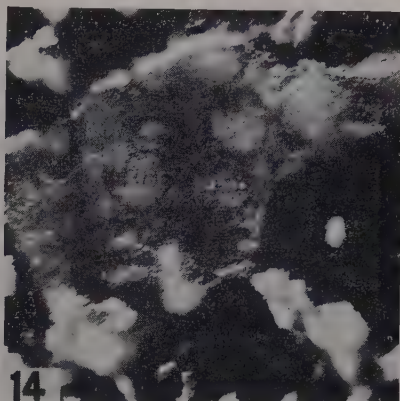
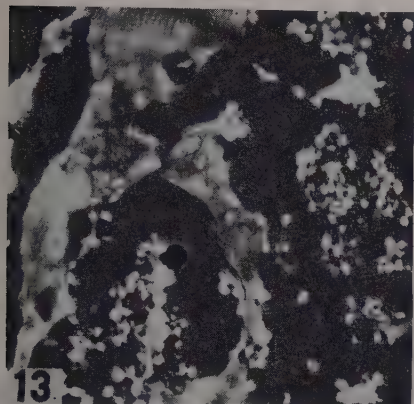


FIGURE 13. Provirus in the cytoplasmic matrix of an infected cell. Mature virus particles are present in the locules which are separated by the provirus containing septa. 3,500 x

FIGURE 14. The provirus or precursor of the virus particle appears to be two or three times the size of the matrix. 14,000 x

FIGURES 15 and 17. Early stages of infection with vaccinia virus with free virus particles in cytoplasm. 2,000 x

FIGURE 16. Sections of virus particles of molluscum contagiosum. 17,500 x

Other examples may be seen in FIGURE 14. All gradations in size and density can be found between mature virus particles and these structures (which are often twice the diameter of a mature virus particle). If provirus is synonymous with "junior-virus" of influenza, then for molluscum "junior" is larger than "father." It appears to us that the mature virus is formed from the cytoplasmic matrix of the cell by a process of segmentation into provirus followed by their condensation into the mature virus. When

an abundant matrix separates a number of small inclusion bodies within a cell, the matrix is often seen undergoing transformation into virus particles. In the more advanced stages, when the inclusions within the cell grow larger, the matrix remains as thin septa, yet within them the same process of segmentation and condensation into virus particles can be observed. It is noteworthy that in early-infected cells, where we can see only a very few virus particles, we have not yet observed segmentation of the matrix in the cytoplasm.

It is interesting to compare our observations with those made in the optical microscope by Rake and Blank.¹⁰ From studies of the molluscum lesion with Feulgen and pyronine methyl-green stains, these investigators have suggested the following sequence during infection of the cell: Masses of deoxyribonucleic acid (DNA) form in the cytoplasm, and these masses compress the cytoplasmic ribonucleic acid (RNA) into trabeculae. From our studies it is apparent that the DNA masses in the cytoplasm are nests of mature virus particles, and these nests are enclosed by walls of varying thickness. These septa are the RNA trabeculae of Rake and Blank. In our electron micrographs, we can see the transformation of these septa into provirus and thence to virus. It follows, therefore, from the cytochemical studies, that in this case we must be observing the conversion of a material rich in RNA to one rich in DNA. As the mature virus is the only cytoplasmic particle definitely associated with the increase in cytoplasmic DNA, the site of conversion may well be in the structure which we have labelled provirus.

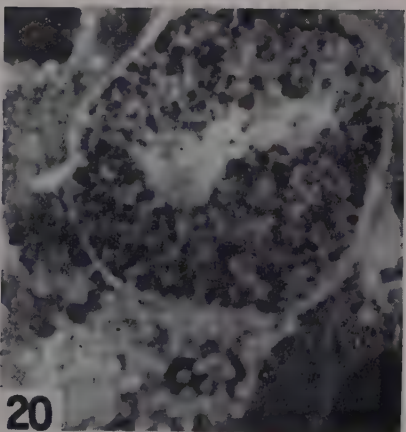
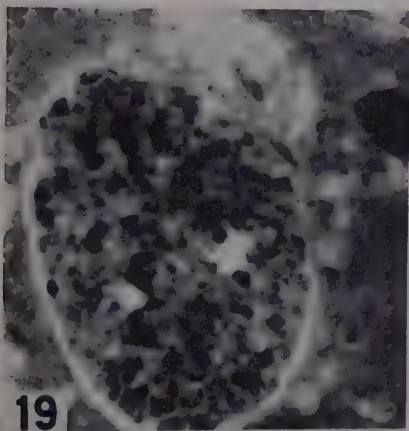
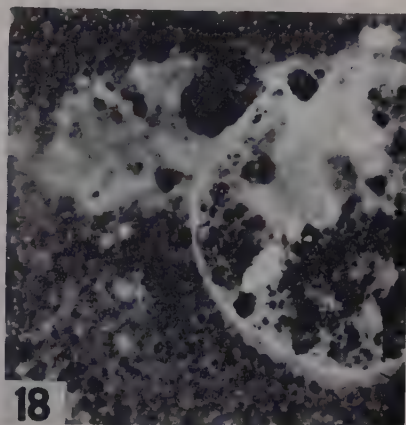
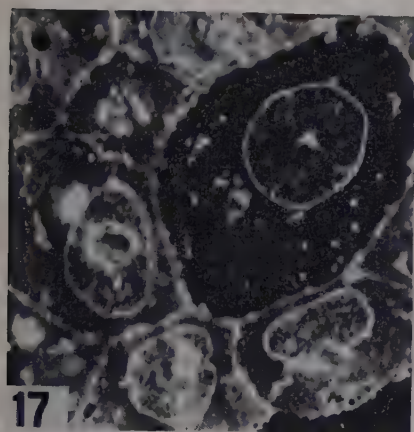
Further observations have been made on the virus particle itself as it exists within the inclusion body of the cell. FIGURE 16 shows a cluster of such virus particles, many of which have had their top and bottom cut off, leaving a dense ring of material with a hollow center. Some virus particles have only been nicked by the knife. That this is a real observation appears certain from the many sections in which both intact and sectioned virus particles are seen. It appears that the virus of molluscum contagiosum contains a formed cortex and a less dense interior.

Vaccinia Virus

Turning briefly to another viral infection of the pox variety which also produces cytoplasmic inclusions, namely that of vaccinia, we should like to emphasize that we have, as yet, never observed any process similar to the transformation of cytoplasm into provirus and thence to virus. Bang¹² and Wyckoff¹³ have recently reported on work in this field. The latter has observed, in infected cells, structures larger than vaccinia virus and has suggested that they may play a role in virus growth. From the numerous sections which have been examined¹¹ we feel that we have been able to follow the life cycle of the virus in the epidermis of the chorio-allantoic membrane of the chick embryo.

FIGURES 15 and 17 illustrate early stages of infection with few virus particles within the cytoplasm. It is often difficult to distinguish the earliest stage of infection from normal cells; for the latter may, at times,

contain occasional structures similar in size to vaccinia virus (about $260 \times 210 \text{ m}\mu$). From slides illustrating the first formation of new virus particles within the cell (FIGURES 18-21), it is apparent that the nucleus is not playing a passive role. New virus particles appear to be intimately associated with the nuclear membrane and, at times, there seems to be a con-

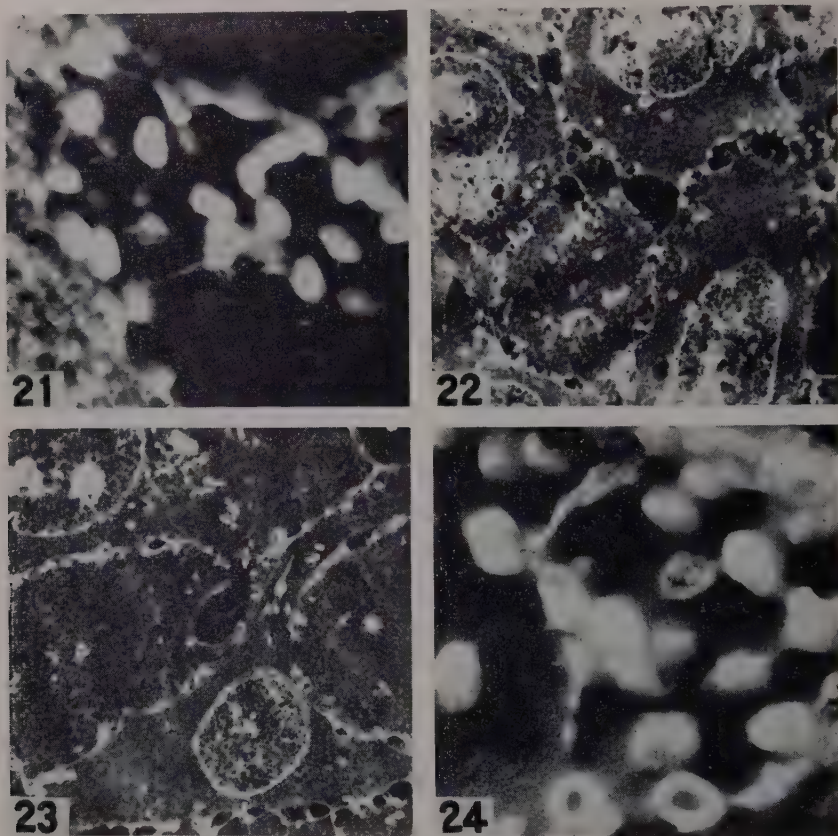


FIGURES 18 to 21. Early formation of cytoplasmic inclusion bodies contiguous with nuclear wall. 5,000 x; Fig. 21: 14,000 x

tinuity of structure from the nuclear membrane to the virus-containing inclusion body within the cytoplasm.

The inclusion body grows larger as it fills up with more and more virus particles and, finally, the cell bursts, liberating the virus into the extra-cellular spaces. We believe that this accounts for those cells which are ringed with virus particles. Relatively few virus particles penetrate such newly-attacked cells (FIGURES 22 and 23). In these cells, however, the events already described take place again, the most striking event of which appears

to be the early formation of virus at the site of, or adjacent to, the nuclear wall. As with molluscum virus, we have produced doughnut shaped viruses by means of the thin sectioning technique (FIGURE 24). Again, it appears that *in situ* another member of the pox virus group may have a cortex



FIGURES 22 and 23. Apparent second cycle in growth of vaccinia virus. Virus liberated in rupture of cells infected in first cycle fill the intercellular spaces, and some particles have penetrated into newly infected cells. Inclusion bodies adjacent to the nucleus are also present in some cells. 2,000 x

FIGURE 24. Sectioned virus particles, some of which appear as doughnuts in view of having both their top and bottom cut off. Others show an internal structure. 23,000 x

more dense than the center of the particle. Sections of some of the virus particles, however, reveal that they may contain internal structure, in agreement with that expected from photographs of the purified virus particles.

In this brief paper we have pointed out how vulnerable to dissection certain virus infections have become. At the same time, they illustrate some of the complexities of the problem. We find that penetration of epidermal cells by two different pox viruses results in two different types of viral growth. In molluscum contagiosum, the cytoplasm itself differentiates

into a provirus phase which condenses and turns into mature virus, often leaving a hole behind in the cytoplasmic matrix. In vaccinia infection, on the other hand, the nucleus seems to play an important role, for the earliest virus particles appear to form at the site of the nuclear wall. At this time, it is not yet possible to predict the type of multiplication of other viruses. Each will have to be studied separately. It appears unlikely, however, that each will be a law unto itself.

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VIRUS-INDUCED TUMORS OF HUMAN SKIN (WARTS, MOLLUSCUM CONTAGIOSUM)*

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The Squibb Institute for Medical Research, New Brunswick, N. J.*

For over forty years it has been known that two benign tumors of human skin, warts and molluscum contagiosum, are caused by an infectious filterable agent.³ Although they are the only proved virus-induced tumors in man, little attention has been paid them until recently. Our ignorance of these viruses is exemplified by our lack of knowledge of the basic immunologic phenomena which occur with infection. It is clear, however, that human warts and molluscum contagiosum have never been successfully transmitted to other than human hosts.

The relatively large virus of molluscum has been well characterized under the electron microscope and is one of the members of the "pox group" of viruses.¹⁰ Recently a small body, forming crystal-like structures, has been demonstrated from several human warts.¹¹

The changes which occur in epithelial cells infected with each of these viruses are unique. The histologic architecture of warts is distinctive, exhibiting a marked increase in the thickness of the several layers of the epidermis (FIGURE 1). The only specific cytologic changes we were able to observe, however, appeared in the nuclei of the epithelial cells. It was reasoned that an accumulation of a distinctive mass of nucleoprotein within a virus-infected cell might be demonstrated microscopically, and thus reveal the presence of a mass of nucleoprotein containing virus particles. Using ultra-violet microscopy Hyden showed, in appropriate areas in the prickle cell layer, the appearance of increasing amounts of nucleic acid within the nuclei of human warts.⁸ All stages in the accumulation of this material can be seen until the nucleus becomes completely filled and distended with a homogeneous mass of nucleoprotein which displaces the chromatin to the periphery of the nucleus. Using the toluidine blue stain for nucleic acids, we confirmed Hyden's studies and also demonstrated with the Feulgen stain that the material was chiefly DNA⁴ (FIGURES 2 and 3). "Inclusion material" in this form, is not seen in any other human skin disease except certain early stages of infection with the herpes simplex and the herpes zoster-varicella viruses.⁶ Other cellular components have been described by earlier workers as virus "inclusion bodies."^{9, 11} Some, however, have confused nucleoli with intranuclear inclusion bodies and others have pointed to the prominent cytoplasmic keratohyalin clumps as inclusion material. Although keratohyalin is markedly increased in warts, it also is seen in normal cells and, in spite of its affinity for hematoxylin, it contains neither DNA nor RNA histochemically. Even though it is prominent in verrucae, its presence cannot be regarded as specific cytologic evidence of infection with wart virus. Additional useful chemical information of the

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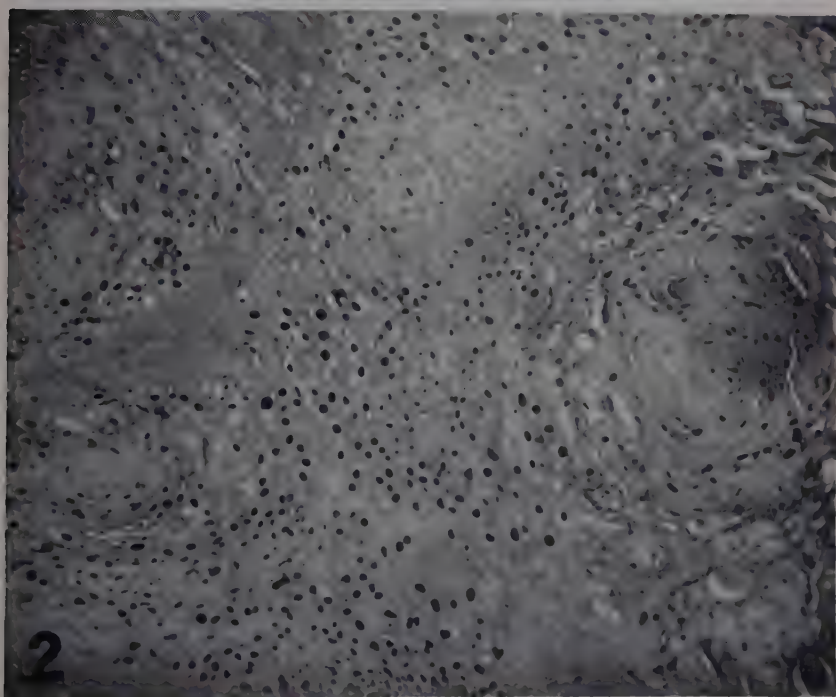


FIGURE 1. Human wart. H & E 75X. The surface of the wart is at the right. There is marked epithelial cell proliferation. The granular layer of the epidermis is thickened with a great increase in keratinized material, some of which has accumulated into large masses within the horny layer.

FIGURE 2. Human plantar wart. H & E 75 X. In this specimen the keratinization is not increased but most of the nuclei have become tilted and somewhat enlarged by a dense mass of intracellular material. Many of these "inclusion bodies" are surrounded by a clear zone producing "bird's eye cells."

nature of verrucae might come from histochemical examination for the large amount of arginase recently demonstrated in these lesions.¹⁵

Many of the cells in the basal and lowermost prickly layers of warts have large, clear nuclei which contain large nucleoli. These cytologic indications of increased cellular activity probably reflect the increased cell proliferation which results in the hyperplastic structure of the wart. It is in these cells that the "colchicine figures" of arrested mitosis and disturbed spindle formation appear, following the application of podophyllin or colchicine to moist warts (*condylomata acuminata*).¹³ The striking biological effect of podophyllin was uncovered by an investigation of the mechanism by which it cures *condylomata acuminata*. That this is a general effect on

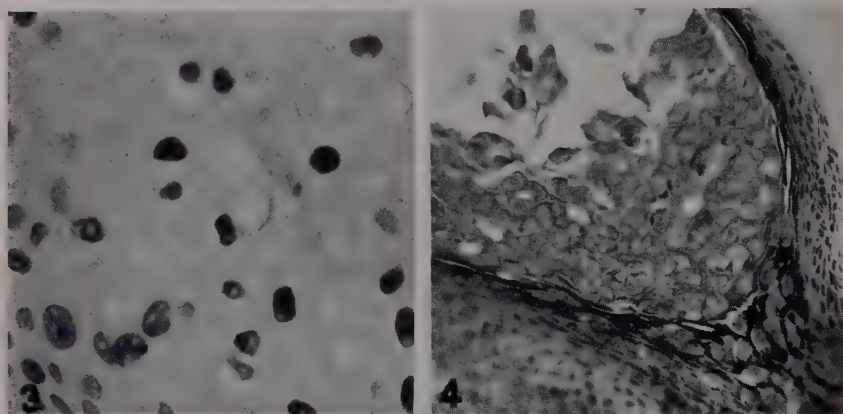


FIGURE 3. Human wart. Feulgen stain 600 X. The transition from relatively normal nuclei with granular chromatin to nuclei, completely filled with DNA staining material is evident. It is not certain that the nuclear vacuoles are the Feulgen-negative RNA containing nucleoli.

FIGURE 4. Human molluscum contagiosum. H & E 250 X. The transition from epithelial cell to "inclusion body" consisting of a cytoplasmic mass of newly formed material displacing the nucleus to the periphery of the cell is evident. (Tissue sectioned from polyethylene glycol media.)

rapidly growing cells rather than the virus itself, is indicated by its ability to affect basal and squamous cell cancers of the skin as well as moist warts.¹²

The cytologic changes which occur in human epithelium infected with molluscum contagiosum resemble those of fowl pox but are not identical with it.⁷ The base of the molluscum lesion is composed of a markedly thickened layer of epithelial cells with intact nuclei containing large nucleoli. As one proceeds to the surface (or core) of the tumor, bizarre changes occur within the cells which reflect successive states of virus multiplication (FIGURE 4). At first, the nucleoli enlarge and the cytoplasmic RNA increases. Within the cytoplasmic RNA, islands of DNA appear which gradually enlarge into distinct compact masses. As the DNA accumulates in large amounts, the pre-existing RNA can be detected only as residual trabeculae (FIGURES 5 and 6). The nucleus is displaced to the periphery of the cell from which it eventually disappears. The final result is a trypsin resistant, carbohydrate encapsulated,¹⁴ DNA-filled body several times larger than the original epithelial cell.^{8, 10}

Electron microscope observations of ground and sedimented molluscum contagiosum material demonstrate an enormous amount of virus in the lesions. In addition to the virus itself, a "sub-virus" particle, approximately

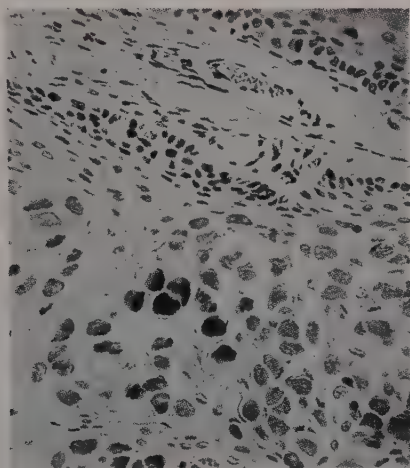


FIGURE 5. Human molluscum contagiosum. Feulgen 167 \times . The cytoplasmic inclusion material contains an enormous amount of DNA. (From Rake and Blank.¹⁰)

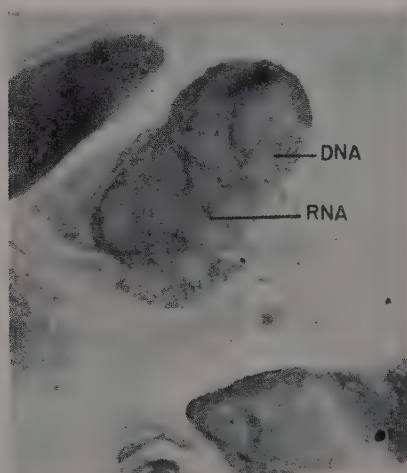


FIGURE 6. Human molluscum contagiosum. Pyronin-methyl green 1200 \times . The residual red staining RNA cytoplasmic trabeculae have almost been replaced by the DNA inclusion material. (From Rake and Blank.¹⁰)

one-fourth the diameter of the virus, has been described. The close association of these particles with fully-formed virus and the lumpy irregular surface of the virus suggest that it may be formed by an aggregation of the smaller particles. Recent genetic studies with other viruses indicate the reasonableness of this theory.

Electron microscopy of thin tissue sections of molluscum lesions prove

the assumption that the large masses of cytoplasmic DNA are actually masses of virus. Further studies are in progress to observe the earliest stages of virus multiplication.¹

Other questions remain to be answered. It is apparent that, at the end stages of infection of an epithelial cell with either the wart or molluscum virus, the cell is far too deranged to undergo any further mitotic division. This is in keeping with the experience with other viruses of the so-called destructive type. It has been demonstrated, however, that "inapparent" infections of cells can occur, even in tissue culture, in which cell proliferation continues in the presence of a "smouldering" virus infection.² It is not known whether this or some other mechanism can account for the proliferative clinical lesion. It might be suggested that the tumor formed is

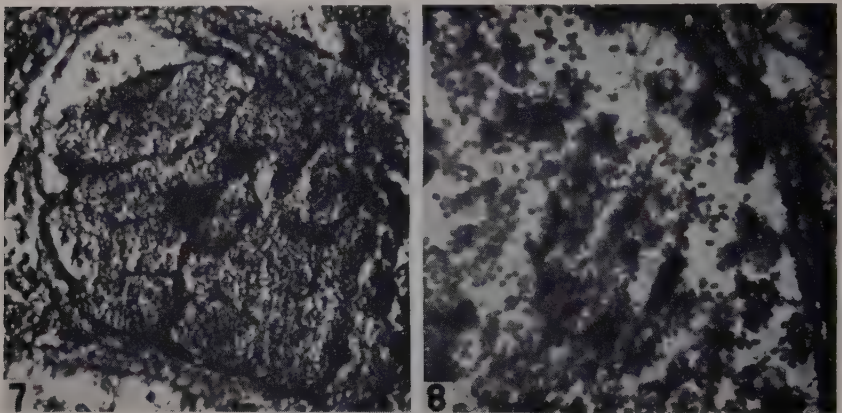


FIGURE 7. Human molluscum contagiosum. Formalin fixed, double embedded. Electron micrograph 6930 \times . The outline of one huge cell can be seen with its trabeculae. The previously seen DNA staining material consists almost entirely of virus particles.

FIGURE 8. Higher power electron micrograph of a lesion similar to FIGURE 7, 10,900 \times . The distinctive virus particles can be distinguished readily. At one edge the carbohydrate containing capsule of the "inclusion body" can be seen as a double walled structure.

actually a "pseudotumor", resulting from a continuous attempt on the part of the host to repair the defect caused by a virus which is slowly destroying epithelial cells. In support of this theory, the production of plaques of proliferated epithelial cells of the chick chorioallantoic membrane by the so-called destructive vaccinia, or herpes simplex viruses, might be considered as an analogous phenomenon. Furthermore, the virus-induced, moderately slowly growing lesions of human skin called "milker's nodules" can be considered as an intermediate type of lesion between a purely destructive vesicle such as smallpox and the proliferative nodule of warts or molluscum. That these viruses do not induce formation of true "tumor cells" might also be suggested from clinical experience for it is clear that malignant transformation of warts or molluscum contagiosum very rarely occurs, unless other stimuli, such as large doses of X-ray therapy sufficient to be carcinogenic in themselves, are added.

The cytologic observations on warts were performed with Dr. Minerva

Buerk, and the electron microscope studies of molluscum contagiosum are part of a study with Dr. Margaret Gray and Dr. Geoffrey Rake.

Conclusions

(1) Infection of human epithelial cells with the wart virus or the molluscum contagiosum virus produce distinctive cytologic changes.

(2) In both diseases these changes result in an accumulation of intracellular inclusion material containing a large amount of desoxyribonucleic acid (DNA).

(3) In warts the specific inclusion material is intranuclear, although there are other accompanying signs of cell proliferation.

(4) Electron microscopy of thin sections of molluscum contagiosum confirms the impression that the cytoplasmic, DNA-containing, inclusion material is a mass of virus particles.

(5) It is apparent that the end stages of infection with these proliferative viruses result in a cell unable to undergo mitotic division and it has been postulated, therefore, that the clinical nodule is a "pseudotumor" produced by the reparative efforts of the epithelium in response to persistent slow destruction of epithelial cells.

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